Historic, Archive Document

Do not assume content reflects current scientific knowledge, policies, or practices.

United States Department of Agriculture

Food Safety and Inspection Service

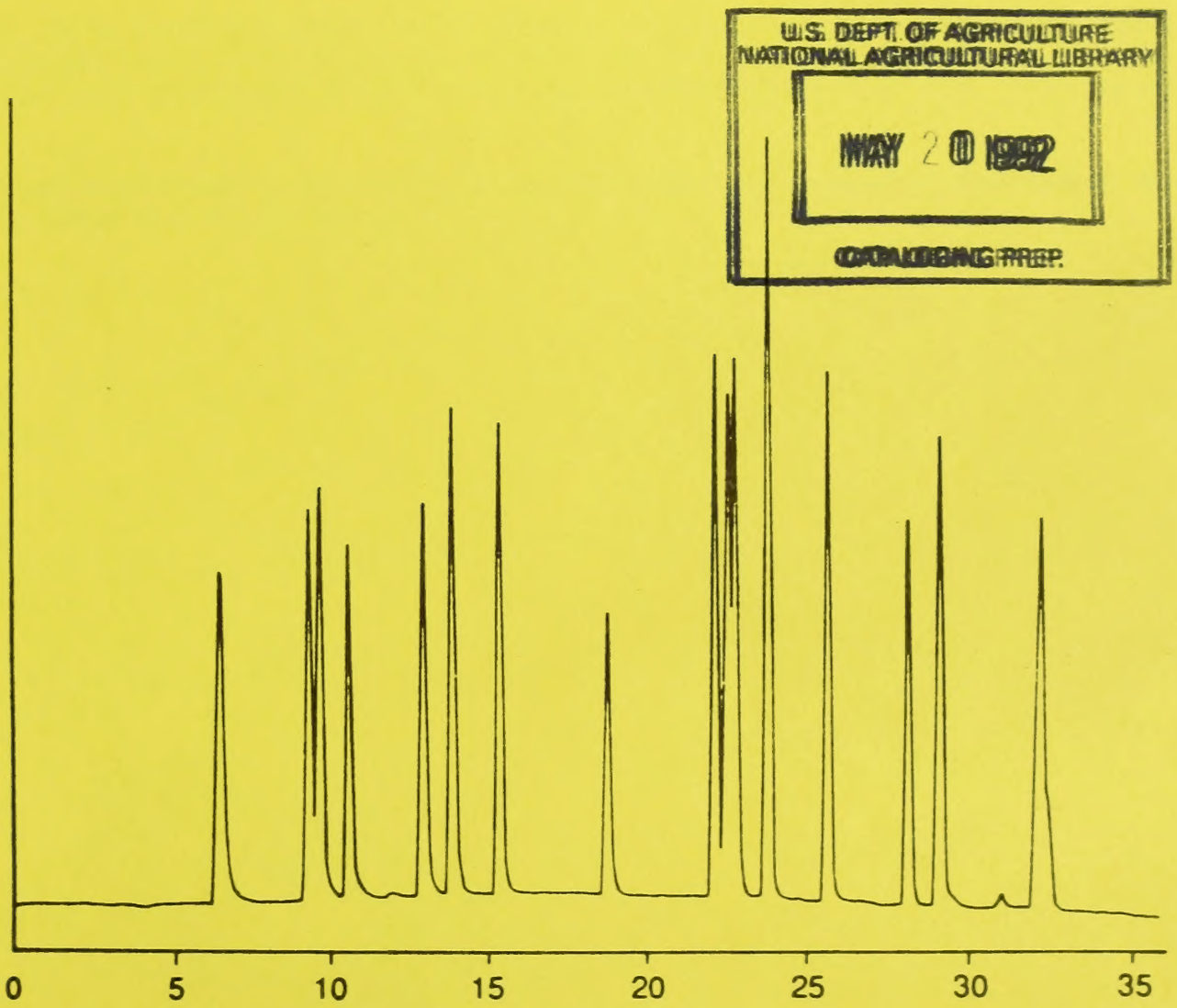
Science and Technology

Winter 1991

Analytical Chemistry Laboratory Guidebook

Residue Chemistry

U.S. DEPT. OF AGRICULTURE
NATIONAL AGRICULTURAL LIBRARY
MAY 20 1992
CATALOGING PREP.



FOREWORD

The new **Analytical Chemistry Laboratory Guidebook** developed jointly by the Chemistry Division and Technical Support Laboratory staffs of the Science and Technology Program is, in the truest sense, a new publication. It is a fresh approach to a user-friendly compendium of analytical methods designed to provide analysts with written documentation to facilitate training, performance, quality assessment and interpretation of observations obtained from these methods to produce high quality analytical results. Safety and quality control/quality assurance matters are an integral part of each analytical method. Everything we have learned or acquired about these laboratory methods is incorporated in a standardized format. For example, samples of analytical chromatograms, mass spectrometry confirmatory data and quality assurance plans are included whenever possible to provide a single source reference for regulatory analysis of meat and poultry products.

The analytical methods recorded here are, to some extent, a reflection of what we have accomplished to provide support to the Food Safety and Inspection Service mission of administering a comprehensive system of inspection laws to ensure that meat and poultry products moving in interstate commerce for use as human food are safe, wholesome and accurately labeled.

The record of our analytical methods attests that this is a dynamic activity, constantly changing, as we update older methods and develop new ones. This development will evolve as we continue to evaluate new technologies and develop them into cost effective regulatory methods. Examples of this are reflected in the new methods incorporated in this volume.

I express my sincere gratitude to those individuals in the Chemistry Division and the Technical Support Laboratories who have contributed heavily to the preparation of this document. This publication has been made possible by the strong support and commitment of the Training and Development Division staff. Their efforts are greatly appreciated.

Lastly, we want your continued involvement in making the **Analytical Chemistry Laboratory Guidebook** the best it can be. Please let us know how we are doing.

Richard L. Ellis
Director, Chemistry Division

Room 302, Annex Building
300 12th Street
Washington, D.C. 20250

TABLE OF CONTENTS

INDEX	METHOD
ALB	ALBENDAZOLE
AMX	AMOXICILLIN
ANA	ANABOLIC STEROIDS
APR	APRAMYCIN
ARS	ARSENIC
ASN	ARSENIC AND TIN
BNZ	BENZIMIDAZOLES
CAM	CHLORAMPHENICOL
CAMU	CHLORAMPHENICOL/URINE
CBM	CARBAMATES
CBX	CARBADOX
CHC1	CHLORINATED HYDROCARBONS
CHC2	HYDROCARBONS/PCB
CHC3	ORGANOHALIDES BY GPC
CHC4	CHLORINATED HYDROCARBON CONFIRM
CLP	CLOPIDOL
CLR	CLORSULON
CRS	CRESYLIC ACID
CYN	CYANIDE
DBT	DIBUTYLTIN
DCQR	DECOQUINATE/ROBOTIC
DEX	DEXAMETHASONE
EDB	ETHYLENE DIBROMIDE
FET	FENTHION
GAS	GASOLINE
GEN	GENTAMICIN
HLF	HALOFUGINONE
IVR	IVERMECTIN
LAS	LASALOCID
LSD	LYSERGIC ACID DIETHYLAMINE
LVD	CYROMAZINE/MELAMINE
MER	MERCURY
MGA	MELENGESTROL ACETATE
MON	MONENSIN
MPT	MORANTEL AND PYRANTEL TARTRATE
MTL	TRACE METAL ANALYSIS
NAR	NARASIN
NBV	NOVOBIOCIN AND VIRGINIAMYCIN
NBVR	NOVOBIOCIN/ROBOTIC
NIC	NICARBAZIN
NTR1	NITROSAMINES/TEA
NTR2	NITROSAMINES/LOW-TEMP VACUUM
ORP1	ORGANOPHOSPHATES
ORP2	ORGANOPHOSPHATE PESTICIDES CONFIRM
PCP	PENTACHLOROPHENOL
PYR	SYNTHETIC PYRETHRINS
PYRE	SYNTHETIC PYRETHRINS/C-ELISA
STY	STYRENE
SUL	SULFONAMIDES
TRZ	CHLORINATED TRIAZINES
ZER	ZERANOL

FSIS

CROSS-REFERENCE
to 1987 Revision

METHOD		NEW INDEX
5.001	CHLORINATED HYDROCARBONS	CHC1
5.002	HYDROCARBONS/PCB's	CHC2
5.003	ORGANOCHLORINE PESTICIDES BY GPC	CHC3
5.004	CHLORINATED HYDROCARBON CONFIRM	CHC4
5.005	ETHYLENE DIBROMIDE	EDB
5.006	ORGANOPHOSPHATES	ORP1
5.007	MERCURY	MER
5.008	ARSENIC	ARS
5.009	ARSENIC AND TIN	ASN
5.010	TRACE METAL ANALYSIS	MTL
5.011	CLOPIDOL	CLP
5.014	CARBADOX	CBX
5.016	FENTHION	FET
5.018-9	SULFONAMIDES	SUL
5.020	NITROSAMINES/TEA	NTR1
5.021	NITROSAMINES/LOW-TEMP VACUUM	NTR2
5.022-3	CHLORAMPHENICOL	CAM
5.024-5	PENTACHLOROPHENOL	PCP
5.026	STYRENE	STY
5.027	GASOLINE	GAS
5.028	LYSERGIC ACID DIETHYLAMINE	LSD
5.029	LASALOCID	LAS
5.030	DECOQUINATE/ROBOTIC	DCQR
5.032	CHLORINATED TRIAZINES	TRZ
5.034	ALBENDAZOLE	ALB
5.035	IVERMECTIN	IVR
5.036	CRESYLIC ACID	CRS
5.037	CYROMAZINE AND MELAMINE	LVD
5.038	CYANIDE	CYN
5.040	MELENGESTROL ACETATE	MGA
5.041	HALOFUGINONE	HLF
5.042	NARASIN	NAR
5.043	GENTAMICIN	GEN
5.044	MONENSIN	MON
5.045	CLORSULON	CLR
5.046	MORANTEL AND PYRANTEL TARTRATE	MPT
5.047	APRAMYCIN	APR
5.048	AMOXICILLIN	AMX
5.049	ZERANOL	ZER
5.050	BENZIMIDAZOLES	BNZ
5.051	ANABOLIC STEROIDS	ANA

ALBENDAZOLES

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 11
G. Calculations 12
H. Hazard Analysis 13
I. Worksheet 14
J. Quality Assurance Plan 17

II. Confirmatory Method

Refer to confirmatory BNZ method.

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Homogenized cattle liver (2.5 g) is fortified with an internal standard [SK&F 101437-C, 5-(Butylsulfonyl)-1H-benzimidazol-2-amine], hydrolyzed in 6N hydrochloric acid, adjusted to pH ≥ 8.0 with sodium carbonate, and extracted with ethyl acetate. The albendazole marker and internal standard are back-extracted into 1N hydrochloric acid and the ethyl acetate removed by aspiration. The aqueous phase is adjusted to pH > 8.0 with sodium carbonate, and then extracted with toluene, which is removed by aspiration. The aqueous phase is applied to a SEP-PAK® C_{18} cartridge where the compounds of interest are retained and eluted with ethyl acetate. The ethyl acetate extract is then concentrated to dryness. The residue is reconstituted in mobile phase, filtered, and quantification performed by HPLC using fluorescence detection.

2. Applicability

This method determines residue concentrations in liver by HPLC, using a fluorescence detector with a lower limit of detection of 125 ppb. Residue findings may be confirmed by GC/MS.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Glass graduated centrifuge tube, 50 mL ground glass stoppered—Kimble 45176-50 or equivalent.

b. Glass graduated centrifuge tube, 13 mL ground glass stoppered—Kimble 45176-13 or equivalent.

c. Oven—Fisher Isotemp Model 176 equipped with thermometer, Fisher Scientific Co., or equivalent.

d. Mechanical shaker, Eberbach flatbed—A. H. Thomas 8287-E30 or equivalent.

e. Centrifuge—Sorvall Model GLC-1, equipped with rotors for 50 mL and 15 mL tubes.

f. N-Evap® —Model 112, Organomation Assoc., Inc., or equivalent.

g. Commercial blender—Waring Model 31BL91 or equivalent.

h. Pipetter—Becton Dickinson Labware (BDL) Model 6606 or equivalent.

i. pH meter—Radiometer Model 22 or equivalent.

j. SEP-PAK® C_{18} cartridges—Waters Assoc. (Part #51910).

k. Vortex-Genie mixer—Model K-550-G, Fisher Scientific Co.

l. Filter assembly—MF-1 Centrifugal Microfilter, Bioanalytical Systems, Inc.

m. Filters, 0.2 μm RC58 (regenerated cellulose)—Bioanalytical Systems, Inc.

n. Balance, top-loading—Sartorious Model 1219MP or equivalent.

o. Dry ice or ice.

p. Pipet, 10 mL glass disposable—A. H. Thomas 7536-H85, Corning #7077, or equivalent.

q. Pipet, Pasteur disposable—A. H. Thomas 7760-A10, 7760-A26, or equivalent.

r. Pipet-Aid® —Drummond Scientific Co.

s. Syringe, 10 mL glass luer tip hypodermic—A. H. Thomas 8932-50 or equivalent.

t. Electrobalance—Cahn Model G-2 or equivalent.

u. Syringe—Model LC-210, Pressure-Lok® , equipped with Rheodyne needle, Precision Sampling Corp., or equivalent.

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

v. Xenon power supply—Model 150, Perkin-Elmer Corp.

w. Vacuum filter, 47 mm glass—Millipore Corp.

x. Filter, Nylon-66 membrane 0.45 μm, 47 mm diameter—Rainin Instrument Co. or equivalent.

y. Syringe, 10 μL glass—Hamilton Model 1801 or equivalent.

z. Syringe, 50 μL—Pressure-Lok®, Precision Sampling Model C-160 or equivalent.

2. Instrumentation

a. HPLC column, μ-BONDAPAK™ C_{18}, 30 cm × 3.9 mm i.d.—Waters Associates P/N 27324 or equivalent.

b. HPLC microprocessor/controller—Altex Scientific Inc. Model 420 or equivalent.

c. Guard column, CO:PELL ODS, 2 mm i.d. × 5 cm—Whatman, Inc., or LiChrosorb 10 μm Brownlee Labs MPLC cartridge or equivalent.

d. HPLC pump—Altex Scientific Inc. Model 100 or equivalent.

e. HPLC injection valve—Rheodyne, Inc., Model 7120 equipped with 20 μL loop or equivalent, or automatic sampler.

f. Recording integrator—Hewlett-Packard Model 5880 or equivalent.

g. HPLC fluorescence spectrophotometer—Perkin-Elmer Corp. Model 204A or equivalent dual monochromator instrument.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hydrochloric acid, concentrated, J. T. Baker Chemical Co., #1-9535, or equivalent.

b. Dimethylsulfoxide (DMSO), distilled in glass, Burdick & Jackson Laboratories, or equivalent.

c. Ethyl acetate, distilled in glass, Burdick & Jackson Laboratories, or equivalent.

d. Methanol, distilled in glass, Burdick & Jackson Laboratories, or equivalent.

e. Toluene, distilled in glass, Burdick & Jackson Laboratories, or equivalent.

f. Acetonitrile, distilled in glass, Burdick & Jackson Laboratories, or equivalent.

g. Diethanolamine, Fisher Scientific Co., D-45, or equivalent.

h. Sodium carbonate, anhydrous powder, Mallinckrodt, Inc., #7521, or equivalent.

i. Water, via Milli-Q/Milli-RO system, Millipore Corp., or equivalent.

j. Potassium phosphate monobasic, Fisher Scientifc Co., P-285, or equivalent.

k. Potassium phosphate dibasic, anhydrous, Mallinckrodt, Inc., #7092, or equivalent.

l. Nitrogen, prepurified grade, M. G. Burdett Gas Products Co., or equivalent.

m. N-Methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), Regis Chemical Co.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. SK&F 81038 5-(Propylsulfonyl)-1H-benzimidazol-2-amine, analytical standard, Smithkline Animal Health Products, 1600 Paoli Pike, West Chester, PA 19380.

b. SK&F 101437, 5-(Butylsulfonyl)-1H-benzimidazol-2-amine, analytical standard, Smithkline Animal Health Products.

2. Preparation of Standards

a. Stock standards.

i. Into a 100 mL volumetric flask, weigh 10.0 mg SK&F 81038 analytical standard (albendazole). Dissolve and dilute to the mark with DMSO (100 μg/mL).

ii. Into a 100 mL volumetric flask, weigh 10.0 mg SK&F 101437 analytical standard (internal standard). Dissolve and dilute to the mark with DMSO (100 μg/mL).

b. Working standards (used for fortifications).

i. Albendazole standard.

(a) Pipet 2.0 mL of stock solution 2.a.i and 8.0 mL DMSO into a 13 mL glass-stoppered centrifuge tube and vortex-mix to produce a 20 μg/mL solution (25 μL fortified to 5.0 g of liver is equivalent to 100 ppb).

(b) Pipet 5.0 mL of working standard 2.b.i.(a) and 5.0 mL DMSO into a 13 mL glass-stoppered centrifuge tube and vortex-mix to produce a 10 μg/mL solution (25 μL fortified to 5.0 g of liver is equivalent to 50 ppb).

(c) Pipet 5.0 mL of working standard 2.b.i.(b) and 5.0 mL DMSO into a 13 mL glass-stoppered centrifuge tube and vortex-mix to produce a 5 μg/mL solution (25 μL fortified to 5.0 g of liver is equivalent to 25 ppb).

(d) Pipet 5.0 mL of working standard 2.b.i.(c) and 5.0 mL DMSO into a 13 mL glass-stoppered centrifuge tube and vortex-mix to produce a 2.5 μg/mL solution (25 mL fortified to 5.0 g of liver is equivalent to 12.5 ppb).

ii. Internal standard.

Pipet 1.0 mL of stock solution 2.a.ii and 9.0 mL DMSO into a 13 mL glass-stoppered centrifuge tube and vortex-mix to produce a 10 μg/mL solution (25 μL fortified to 5.0 g of liver is equivalent to 50 ppb).

I. DETERMINATIVE METHOD

D. STANDARDS (Continued)

c. External standards.

i. Into four glass-stoppered 15 mL tubes, pipet 100 μL of 10 μg/mL internal standard solution into each of the tubes.

ii. To tube A, also add 200 μL of 5.0 μg/mL solution.

iii. To tube B, also add 100 μL of 5.0 μg/mL solution.

iv. To tube C, also add 50 μL of 5.0 μg/mL solution.

v. Tube D has no albendazole added to it (equivalent to 0.0 ppb albendazole).

vi. Evaporate to dryness on N-Evap and bring to volume with 2.0 mL 70/30 water/methanol solution.

vii. Mix and wait approximately 30 minutes to transfer for LC injection.

3. Storage Conditions All standard solutions should be stored in a refrigerator (4° C).

4. Shelf Life Stability Standard solutions are stable in DMSO for a minimum of six months. Because DMSO solidifies under these conditions, it is necessary to reliquefy the standard solutions by placing the tubes in a beaker of lukewarm water and vortex-mix the solutions prior to use.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Representative sections of cattle liver are thoroughly homogenized in a commercial Waring Blender until no visible pieces remain (approximately 3-5 minutes/100 g). Homogenates should be kept frozen at all times until analyzed.

NOTE: If frozen cattle liver is used, it must be kept frozen until assay. Liver homogenate stored in small vials is thawed under lukewarm water prior to each assay. Brief thawing of either the frozen liver or frozen liver homogenate is acceptable, if refrozen as soon as possible.

2. Sample Extraction

a. Into the bottom of a 50 mL glass centrifuge tube (unstoppered), supported in a beaker tared on a balance, weigh 5.0 ± 0.1 g homogenized liver, using a wide-tip disposable pipet. Prepare four blank tissue samples, one for control and three for fortification. Add 100 μL of pure DMSO to the control. Add 25 μL DMSO to the tissues suspected of containing albendazole residue. Add 25 μL of working standard solution 2.b.ii to each of the remaining three blank tissue samples and the tissue samples suspected of containing albendazole residue. Add 50 μL of working standard solutions 2.b.i.(a), 2.b.i.(b), and 2.b.i.(c) to separate blank tissue samples from the set of three already fortified with working standard solution 2.b.ii.

   NOTE: Fortification solutions should be added to the 50 mL tube just above the homogenate to ensure solution when the HCl is added in the next step.

b. Add 5.0 mL of 6N HCl to each sample prepared in step 2.b, stopper each tube, and place the sample tubes (covered with another tube rack to prevent stoppers from popping) in an oven preset to 110° C ± 4° C (at elevations ≤ 1000 ft above sea level).

   NOTE: At elevations > 5000 ft, hydrolysis for 30 minutes in a 15 psi pressure vessel is required to adjust for atmospheric pressure. Timing for hydrolysis should begin when the samples are placed into the pressure vessel with the water already boiling. It is necessary to have the water boiling to reach water pressure as quickly as possible (≤ 4 minutes). The hydrolysis time is critical and must be timed accurately.

c. After 1 hour, remove the samples from the 110° C ± 4° C oven and allow the tubes to cool to room temperature over dry ice or ice for approximately 5 minutes.

d. Add 2.0 mL water (Milli-Q) to each sample. While vortex-mixing the sample, slowly add enough powdered sodium carbonate (approximately 1.23 g over 4-5 minutes to avoid foaming or caking) to achieve a pH of 8-9.5 when monitored with a pH meter. (Place the clean pH electrode directly into the sample. After recording the pH, dab any drop of solution from the electrode on the inside of the tube. Completely remove the electrode from the tube, rinse it with water, and dry it with a Kimwipe before monitoring the next sample.

   NOTE: Samples at or near room temperature do not foam as much as colder samples. Therefore the Na_2CO_3 can be added faster.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

e. Add 10.0 mL ethyl acetate to each sample and stopper the tubes. Shake the samples, supported in a rack in a horizontal position, for 5 minutes. Remove the stoppers and centrifuge the samples at approximately 400 G for 5 minutes.

f. Transfer as much as possible of the ethyl acetate extracts to separate 50 mL glass centrifuge tubes, using a disposable pipet and *taking care not to transfer any of the aqueous phase*.

NOTE: The Pipet-Aid®, manufactured by Drummond Scientific Inc., is a convenient time-saving device for transferring or aspirating solutions.

g. Reextract each sample a second time by repeating steps e and f and pooling the appropriate extracts in the respective 50 mL glass centrifuge tubes.

NOTE: A $\geq$ 75% recovery is achieved by taking care to transfer as much as possible of the extraction solvents in steps e, f, and g.

h. Add 4.0 mL of 1N HCl to each ethyl acetate pooled extract and stopper the tubes. Shake the samples, supported in a rack in a horizontal position, for 5 minutes. Remove the stoppers and centrifuge the samples at approximately 400 G for 5 minutes.

i. Remove as much as possible of the ethyl acetate phase by aspiration, *taking care not to remove any of the aqueous phase*. Evaporate the remaining ethyl acetate ($\leq$ 1 mL) from the aqueous phase in the N-Evap® waterbath at 35° C $\pm$ 5° C.

Stop point: If necessary, the extraction can be stopped at this point overnight.

j. While vortex-mixing each sample, add enough sodium carbonate slowly (approximately 0.33 g over 1-2 minutes to avoid foaming or caking) to achieve a pH of 8-9.5 when monitored with a pH meter.

k. Add 15.0 mL toluene to each sample and stopper tubes. Shake the samples, supported in a rack in a horizontal position, for 5 minutes. Remove the stoppers and centrifuge the samples at approximately 400 G for 5 minutes.

l. Remove as much as possible of the toluene phase by aspiration, *taking care not to remove any of the aqueous phase*. Evaporate the remaining toluene ($\leq$ 1 mL) from above the aqueous phase in the N-Evap® waterbath at 35° C $\pm$ 5° C.

Stop point: If neccessary, the extraction can be stopped at this point overnight.

m. Attach a SEP-PAK® C_{18} cartridge to the luer tip of a 10 mL glass syringe, secured to a ring stand with a clamp, for each sample (cylinder barrel removed).

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

n. Prewash each cartridge with 2 mL methanol followed by 2 mL of 0.2M potassium phosphate buffer, pH 8.0. Each reagent is transported through the cartridge by means of gentle nitrogen pressure applied through a rubber stopper fitted into the top of the syringe after addition of the respective reagent. The nitrogen pressure is removed when nitrogen begins to exit the cartridge. Flow rate approximately 1-2 mL/min. Discard eluates.

o. Transfer the aqueous phase from each sample from step l to a separate prewashed syringe cartridge unit. Elute this phase from the cartridge using nitrogen as in step n, then transfer a 1 mL water (Milli-Q) wash of each sample tube (vortex-mixed) to the respective syringe-cartridge unit in a similar manner using the respective Pasteur pipet. Discard all eluates.

NOTE: Transferring the 4 mL aqueous phase should be done quantitatively using a Pasteur pipet. The 1 mL wash of the sample tube is transferred to the SEP-PAK® C_{18} cartridge using the same Pasteur pipet. When transferring solutions, separate disposable and Pasteur pipets are used for each sample to avoid contamination.

p. Add an additional 1 mL water (Milli-Q) wash of each sample tube (vortex-mixed) to the respective syringe-cartridge unit and elute using nitrogen as in step n. Discard eluates.

q. Add 2 mL toluene to each syringe-cartridge unit and elute using nitrogen as in step n. Discard eluates.

r. Place a 13 mL glass centrifuge tube beneath each syringe cartridge unit. Add 2 mL ethyl acetate to each unit and elute using nitrogen as in step n. This fraction contains the compound of interest.

s. Add about 0.5 mL methanol to each eluate from step r and vortex-mix to obtain a homogeneous solution.

t. Concentrate the eluates from step s to dryness under dry nitrogen in an N-Evap®waterbath at 35° C ± 5° C.

u. After reaching dryness, add 0.5 mL of the water/methanol (70/30) solution and vortex-mix to reconstitute the residue.

NOTE: The sample extracts contained in water/methanol (70/30) are stable for at least a month and if reanalyzed provide data similar to the previous HPLC analysis.

v. After > 30 minutes, apply each of the reconstituted samples to a separate Bioanalytical System filter unit fitted with an 0.2 μm regenerated cellulose filter. Centrifuge each filter unit containing a sample extract at approximately 400 G for 5-10 minutes.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

w. Transfer the filtrates to separate 13 mL glass-stoppered centrifuge tubes and adjust the volume of each filtrate to 0.5 mL with water/methanol (70/30). Use of a silanized tube is helpful to concentrate the extract residue to the very bottom of the tube. After vortex-mixing, remove 0.1 mL for HPLC analysis. See Confirmation Method, section E, for disposition of remaining 0.4 mL liver extract samples.

3. Verification of Control Cattle Liver

To determine that a cattle liver can be used as a control, proceed as follows: Prepare four 2.5 g liver homogenate samples. Fortify two aliquots with 100 μL DMSO and two aliquots with 50 μL DMSO and 50 μL of the internal standard solution 2.b.i.(d) (200 ppb). The samples are processed through the extraction method and analyzed using the HPLC procedure. The nonfortified control samples (DMSO only) should produce either no or only a negligible response at the retention time corresponding to the internal standard as compared to the response observed for a known control sample fortified at 200 ppb. Providing this background is negligible, calculate the background at the retention time corresponding to the albendazole marker in the control samples fortified with internal standard. If a response is observed, calculate the peak area (height) ratio. If either no response or peak area (height) ratio is less than 5% of that calculated for a known control fortified at 200 ppb is determined, the cattle liver is considered a control.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Instrumental Settings and Conditions

a. Column.

30 cm × 3.9 mm I.d. μ-BONDAPAK™ C_{18}.

b. Flow rate.

1.8 mL/min (Bondapak), 1.0 mL/min (Alltech).

c. Mobile phase.

i. 60% 0.02M KH_2PO_4; 0.01M diethanolamine (200 mL 0.1M KH_2PO_4 + 1.05 g diethanolamine taken to 1 L with Milli-Q water).

ii. 30% methanol.

iii. 10% acetonitrile.

This solution is prepared daily, filtered through a 0.45 μm Nylon-66 filter, and degassed under vacuum prior to use.

d. Fluorescence detector.

Initially set the excitation and emission wavelengths at 300 nm and 320 nm respectively, with a slit width of ≤ 10 nm for both. Inject an appropriate amount of sample (20 μL) and observe the intensity of the response for both SK&F 81038 and SK&F 101437 with a reasonable baseline sensitivity setting. If additional sensitivity is required, proceed as follows:

While observing the detector absorption response, adjust the excitation wavelength to ≤5 nm to reduce the zero suppression. Reinject the same sample as above and determine if the signal responses for the compounds of interest increase. Continue this procedure of optimizing both the excitation and emission wavelengths to obtain an adequate signal to noise ratio for the analysis (s/n ≥ 25/1).

e. Temperature.

Column, mobile phase, and detector temperature = ambient (70° ± 2° F).

f. Approximate retention time.

i. Albendazole marker (SK&F 81038): approximately 4.3 minutes.

ii. Internal standard (SK&F 101437): approximately 7.0 minutes.

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Divide the peak area of the albendazole marker SK&F 81038 by the peak area of the internal standard SK&F 101437 from the HPLC data for each sample extract to obtain peak area ratios.

$$\text{peak area ratio} = \frac{\text{area of albendazole marker peak (SK\&F 81038)}}{\text{area of internal standard peak (SK\&F 101437)}}$$

b. Construct an analytical curve by plotting, on linear axes, the peak area ratio versus the respective ppb value for each fortified control sample. Using the regression equation for the analytical curve, directly calculate the ppb level of albendazole marker in the unknown samples from their peak area ratios using the following equation:

$$x = \frac{y - b}{m}$$

where x = ppb concentration in suspect samples

y = peak area ratio of suspect sample extract

b = intercept from regression equation

m = slope from regression equation

NOTE: If an integrator is unavailable, peak height measurements and peak height ratio data can be substituted for peak area and peak area ratio data in the above computation.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination and Confirmation of Albendazole Residues in Animal Tissue

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat, fume hood.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Dimethylsulfoxide	Noxious irritant with vapors that permeate an area very readily.	Analytical standards requiring this solvent must be prepared in an efficient fume hood.
Ethyl acetate	Corrosive solvent with vapors that may cause irritation to the eyes, skin, and respiratory system.	Use only in an efficient fume hood.
F. Mobile phase		
KH_2PO_4 Diethanolamine Methanol Acetonitrile	Corrosive noxious mixture.	The mixing, filtering, and degassing should be done in an efficient fume hood.

4. Disposal Procedures

Organic solvents	See above	The volumes may be reduced by partial evaporation. The remainder should then be safely stored until disposal by contractor or in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Albendazole Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: ALBENDAZOLE ANALYSIS

Analyst: _____ Analyst Code: _____ Date: _____

EXTERNAL STANDARD CURVE DATA

Standard	μg/mL	Ratio of Albendazole to Int. std
D	0	
C	.125	
B	.250	
A	.500	

REGRESSION ANALYSIS

b	
m	
r	
s	

Sample Size: 5.0 g.

Mobile Phase: 60% 0.02 Phosphate Buffer, 30% MeOH, 10% Acetonitrile

Flow Rate: 1.0 mL/min

Column: Alltech 25 cm × 4.1 mm id Versapack C_{18} 10 μ #103084-13, or equivalent.

SAMPLE IDENTIFICATION	CRITICAL CONTROL POINTS								
PROCEDURE KEY	Sample Weight (g)	μL of Internal Standard	First Addition Na_2CO_3 (g)	Second Addition Na_2CO_3 (g)	Temp. H_2O Bath (°C)	Final Volume (mL)	Ratio Albendazole Int standard	Albendazole Found (ppb)	COMMENTS
FORTIFIED CONTROL 25 ppb Marker Compound									

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability % CV*
Albendazole	≥ 20	60-100[1]	<20

[1]After correction for tissue/reagent background.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5.0 ± 0.1 g
b. Internal standard volume	50 μL
c. Standard curve volume additions	50 μL each standard
d. pH of samples (E.2.d, E.2.j)	8-9.5 pH
e. Extract dissolution	0.5 mL methanol/water

3. Readiness To Perform

a. Familiarization.

i. Phase I: Acceptable standard curves for 3 successive days.

ii. Phase II: Duplicate tissue recoveries on 3 successive days at 0, 12.5, 25, and 50 ppb.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 bovine liver samples fortified between 0 and 50 ppb submitted by FSIS Science & Technology Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report data to Chemistry Division.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Initially, at least one check sample monthly per analyst.

ii. Blind samples or random replicates chosen by the supervisor and/or the Laboratory QA Officer.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All recovery values.

(b) Running average of 10 latest acceptable recovery values.

(c) CUSUM control chart on recovery values.

iv. Records (supervisor):

(a) Record of blind check samples.

(b) CUSUM of blind check samples.

(c) Standards book.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Liver.

b. Sample receipt size: Varied; enough to obtain matrix required for all quantitative and confirmation tests.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: $\leq$ 3 months.

ii. Condition: frozen.

AMOXICILLIN

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 7
G. [Reserved]
H. [Reserved]
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Amoxicillin is extracted from beef and swine tissues with water, and potentially interfering substances are removed by precipitation with trichloracetic acid and by extraction with diethyl ether. Amoxicillin is converted to a fluorescent compound by heating in an acid medium, after which it is detected using an HPLC with fluorometric detector. Amoxicillin levels of 10 ppb can be detected by this method.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Tissue homogenizer: Tekmar SDT, or equivalent, equipped with small homogenizing shaft.

b. Centrifuge: Damon/IEC model PR-6000 or equivalent.

c. 50 mL polypropylene centrifuge tubes: Becton Dickinson, 1950 Williams Drive, Oxnard, CA 93030, or equivalent.

d. Mohr pipets: 10 mL.

e. Volumetric pipets: 1 and 2 mL.

f. Repipet dispensers: 10 and 25 mL.

g. Vortex mixer.

h. Oil bath: Fisher Hi-Temp Bath, or equivalent.

i. Glass beads: Approximately 3 mm diameter.

j. Analytical evaporator: N-Evap, manufactured by Organomation, Inc., P.O. Box 159, South Berlin, MA 01549.

k. Glass Luer-Lok syringe: 10 mL, equipped with 18 gauge stainless steel 5″ needle.

l. Volumetric flasks: 100 mL.

2. Instrumentation

a. μ-Porasil HPLC column: 30 cm × 3.9 mm, 10 micron packing (Waters).

b. 100 microliter syringe: Compatible with HPLC U6K injector (Hamilton Co.).

c. HPLC: Spectra-Physics 3500B, equipped with Waters U6K injector or equivalent.

d. Fluorescence spectrophotometer: Perkin-Elmer 650-10S or equivalent.

e. Strip chart recorder: Fisher Recordall Series 5000 or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Trichloroacetic acid—ACS reagent grade.

b. Anhydrous diethyl ether—ACS reagent grade.

c. Formaldehyde solution, 7% w/v—prepared by dilution of 37% certified ACS grade of formaldehyde solution.

d. Mineral oil (for heating bath).

e. Hydrochloric acid—ACS reagent grade.

f. Potassium chloride—ACS reagent grade.

g. Methylene chloride—distilled in glass (Burdick & Johnson or equivalent).

h. Methanol—distilled in glass (Burdick & Johnson or equivalent).

i. Buffer—pH, 2.0 (375 mg KCl plus 1.3 mL of 1.0N HCl, diluted to 100 mL with distilled water).

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Amoxicillin—crystalline.

Sigma Chemical Co.
P.O. Box 14508
St. Louis, MO 63178

2. Preparation of Standards

a. Amoxicillin standard solution.

i. Weigh 100 mg amoxicillin into a 100 mL volumetric flask. Dilute to the mark with distilled water and mix thoroughly by inversion. This is the 1,000 μg/mL stock standard solution.

ii. Take 1.00 mL of the amoxicillin stock solution, pipet it into a 100 mL volumetric flask, dilute to the mark with distilled water, and mix thoroughly by inversion. This is the 10 μg/mL working standard solution.

b. Amoxicillin derivative for retention time.

i. To a 50 mL polypropylene centrifuge tube, add the following:

(a) 200 μL of the 10 μg/mL amoxicillin standard solution.

(b) 3 mL of pH 2.0 buffer.

(c) 1.5 mL of 7% formaldehyde solution.

(d) 10 mL distilled water.

ii. After vortexing the tube contents, heat the solution in an oil bath at 95° C for 2 hours and 20 minutes.

iii. Cool in an ice water bath for about 5 minutes.

iv. Add 10 mL methylene chloride/methanol (95/5) and shake vigorously for 1 minute.

v. Centrifuge 5 minutes at 3500 rpm at room temperature.

vi. Aspirate and discard the top (aqueous) layer.

vii. Inject an aliquot of the methylene chloride/methanol solution into the HPLC to give a peak that will serve as a retention time marker for amoxicillin.

NOTE: If it is not possible to make injections immediately, transfer the tube contents to a glass container and hold in a refrigerator.

c. Reagent blank.

A reagent blank is prepared by substituting 15 mL distilled water in place of the 15 mL of tissue extract and treating similarly. Amoxicillin should not be detectable under these conditions.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

a. Weigh 15 g frozen, previously blended tissue into a 50 mL polypropylene centrifuge tube.

b. Weigh two 15 g portions of tissue known to be free of amoxicillin into each of two 50 mL polypropylene centrifuge tubes. To one portion add 15 μL of the 10 μg/mL amoxicillin standard (level of this spike = 0.010 ppm = 10 ppb). The other portion serves as a method blank.

c. Add 20 mL distilled water to each tube and homogenize contents for 3 minutes at moderate speed. (Clean tissuemizer head thoroughly between samples.)

d. Centrifuge the tubes for 30 minutes at 3500 rpm at room temperature.

e. Decant supernatants into a second set of 50 mL polypropylene centrifuge tubes.

f. Add 1.5 g of trichloroacetic acid to each of the tubes, cap, shake vigorously 1 minute, and cool in an ice bath for 5 to 10 minutes.

g. Centrifuge for 10 minutes at 3500 rpm at room temperature.

h. Decant supernatants into a third set of 50 mL polypropylene centrifuge tubes, add 15 mL of diethyl ether to each, cap, and shake *very gently* for 20 seconds (use a ''bicycle'' motion to gently mix the tube contents).

i. Centrifuge for 10 minutes at 3500 rpm at room temperature.

j. Aspirate upper layer (ether) and discard.

k. To each tube, add 1 mL of 20% trichloroactic acid solution, 3 mL of pH 2.0 buffer solution, and 1.5 mL of 7% formaldehyde solution.

l. Add several glass beads (pre-cleaned with ether) to each tube. Vortex the tube contents and place in a mineral oil bath at 95° C for 2 hours and 20 minutes.

   i. Leave caps off the tubes until residual ether boils off; then screw caps on loosely.

   ii. Perform this step in an efficient fume hood.

m. Cool tubes in an ice water bath for 5 minutes.

n. Add 15 mL diethyl ether to each tube and shake *very gently* for 2 minutes.

o. Centrifuge for 10 minutes at 3500 rpm at room temperature.

p. Using a glass syringe fitted with a 5″ needle, aspirate the bottom (aqueous) layer of each tube and discard. Clean the syringe and needle thoroughly between each sample.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

q. Evaporate tube contents under a stream of nitrogen on an N-Evap set at 60° C.

r. Reconstitute by adding 2.0 mL methylene chloride/methanol (95/5) to each tube and mix thoroughly, using vortex mixer.

NOTE: If it is not possible to make injections into the HPLC immediately, transfer the reconstituted tube contents to glass containers and hold in a refrigerator.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

Chromatographic conditions.

a. Column: μ-Porasil (Waters), 30 cm × 3.9 mm i.d.

b. Flow: 1.0 mL/min.

c. Mobile phase: Methylene chloride/methanol (95/5).

d. Fluorescence detector: Perkin-Elmer 650-10S or equivalent.

e. Excitation wavelength: 362 nm.

f. Emission wavelength: 435 nm.

g. Attenuation range: 30 with a 10mV recorder (vary this if more or less sensitivity is needed).

h. Slits: 7 nm.

i. Injection volume: 50 μL (vary this if more or less response is needed, but the *same volume* should be injected for each sample and for the spiked and blank tissue extracts).

j. Retention time: 9 minutes (approximately); measure exactly with the amoxicillin retention marker derivative.

2. General Operation

Determination of the level of amoxicillin residue: If the peak height of the tissue sample is less than ½ that of the peak height of the sample spiked at 0.01 μg/g (10 ppb), report that the tissue contains less than 0.01 ppm or less than 10 ppb of amoxicillin.

3. Reference

NADA 55-080 and 55-089 Beecham.

ANABOLIC STEROIDS

Contents

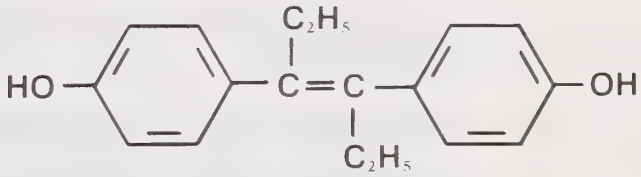
A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 11
G. Calculations 14
H. Hazard Analysis 15
I. Worksheet 16
J. Quality Assurance Plan 19

DETERMINATIVE/CONFIRMATORY METHOD

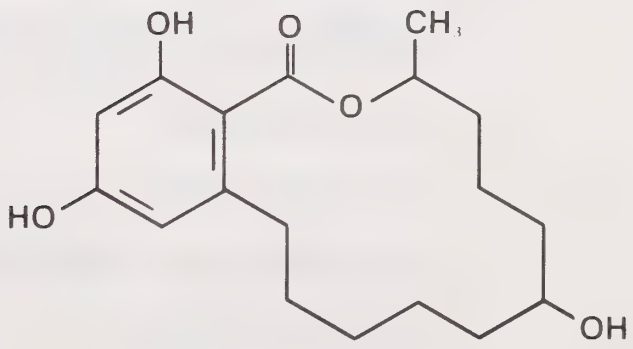
A. INTRODUCTION

1. Theory and Structure

This procedure uses a three-phase solvent extraction of liver using an aqueous buffer, acetonitrile, dichloromethane, and hexane to effectively remove the majority of the triglycerides and highly nonpolar materials prior to loading on the column. This step prevents the overloading of the column, which would reduce recoveries, and simultaneously fractionates compounds into three polarity classes. An extraction cartridge containing a strongly basic anion exchange resin with quaternary amine groups in the chloride form is used to extract the DES and zeranol. The phenolate anion of these compounds has a high affinity for the chloride form of the resin at pH values above 12.0. The acetonitrile extract of the tissue is concentrated, made alkaline, and the analyte is exchanged to the resin. Several solvent washes are performed to remove interfering substances. A 5% aqueous acetic acid wash allows for the elution of a large quantity of interfering anionic materials previously exchanged to the resin. The column is then eluted with a solution of limited solvent strength (25% aqueous methanol) to remove residual acetic acid and additional matrix material prior to the final elution with methanol. The procedure is amendable to automation using the four-solvent Dupont Prep I centrifugal column elution device, which significantly increases sample throughput.



DIETHYLSTILBESTROL
($C_{18}H_{20}O_2$; mol wt 268.34)



ZERANOL
($C_{18}H_{26}O_5$; mol wt 322.41)

2. Applicability

This method will detect DES, zeranol, and taleranol in ovine and bovine liver and muscle.

DETERMINATIVE/CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus

a. 50 mL screw-cap polypropylene centrifuge tubes (Falcon, or equivalent).

b. Homogenizer—Tekmar SDT Tissuemizer with 1810 motor and SDT-100EN shaft and generator and TR-10 high torque speed control, or equivalent.

c. Centrifuge—IEC PR-6000, equipped with 12-place angle rotor, or equivalent, and capable of achieving a G force of $\geq$ 2500.

d. Pasteur disposable pipets.

e. 50 mL glass-stoppered (standard taper) centrifuge tubes.

f. Repipet dispensers, 10 mL (Labindustries, Inc., or equivalent).

g. Low temperature indicator (Precision Scientific, Freas Model 815), or constant temperature water bath (35°-37° C), or equivalent.

h. Type AS anion exchange cartridge.

i. Plastic recovery cups.

j. Prep I automated sample processor—Dupont Chemical Co., catalog #60000-908.

k. Scintillation vials, 20 mL (Kimble #74500, or equivalent).

l. Reacti-Therm dry block heater with custom-drilled heat blocks (Pierce Chemical Co., or equivalent).

m. Fisher sample concentrator, Model 190, custom-drilled to accept 20 mL scintillation vials (catalog #09-549), or equivalent.

n. Vortex mixer.

o. Tapered 1 mL auto sampler vials, 1 CWVC combo-pack and 8 pep-6 polyethylene plug (Chemical Research Supplies, or equivalent).

p. Eppendorf pipets.

q. Disposable pipet, 10 mL.

r. pH meter—Orion Model 601A, or equivalent.

s. Syringe—100 μL.

t. Rotorack or Eberbach shaker (optional).

2. Instrumentation

GC-MS—Hewlett-Packard, 5890 GC, 5970A series mass selective detector and 9825B computer, or equivalent.

DETERMINATIVE/CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Acetonitrile, hexane, dichloromethane, methanol, ethyl acetate, isopropyl alcohol—Burdick & Jackson, high purity solvent grade, or equivalent.

b. Water—distilled, deionized.

c. Acetic acid—glacial, USP #9522, Canlab Chemicals, or equivalent.

d. Derivatizing agents—BSTFA, Pierce Chemical Co., #38830; TMSI, Pierce Chemical Co., #88623.

e. Sodium hydroxide—reagent grade pellets, #3722-1, J. T. Baker Chemical Co., or equivalent.

f. 2N Sodium acetate—Fisher Certified Reagent, #S209B, Fisher Chemical Co., or equivalent.

g. 2N Sodium hydroxide—80 g/L in distilled, deionized water.

h. 1:1 Isopropanol:methanol—100 mL of each, measured separately and mixed.

i. 5% Aqueous acetic acid—5 mL glacial acetic acid made up to 100 mL with distilled, deionized water.

j. 25% Aqueous methanol—25 parts methanol; 75 parts distilled, deionized water; measured separately and mixed.

k. 0.04M Sodium acetate buffer—5.44 sodium acetate dissolved in 1 L distilled, deionized water.

l. β-Glucuronidase—Type H-25, Sigma Chemical Corp., St. Louis, MO. Activity approximately 100,000 units/mL.

DETERMINATIVE/CONFIRMATORY METHOD

D. STANDARDS

1. Source

a. Zearalane—zeranol assay internal standard. Not commercially available. Can be obtained by request from Pitman-Moore, 1401 S. 3rd St., Terre Haute, IN 47802.

b. Zeranol, taleranol—Not commercially available. Can be obtained by request from Pitman-Moore.

c. D_8DES—DES internal standard (diethyl-1,1,1′,1′-d_4-stilbestrol-3,3′,5,5′-d_4), MSD Isotopes (Merck, Sharpe & Dohme Isotopes), Montreal, P.Q.

d. DES—99% purity, #21894-4, Aldrich Chemical Co.

e. Diethylstilbestrol monoglucuronide (DES-MG)—#16444-5, Aldrich Chemical Co. Each ampoule contains the equivalent of 1 mg of free DES.

2. Preparation of Standards

a. DES, D_8DES, taleranol, zearalane, zeranol.

i. Stock solutions: Weigh 20 mg each of taleranol, zearalane, and zeranol and 10 mg each DES and D_8DES into separate 100 mL volumetric flasks. Dilute to volume with methanol (concentration 200 μg/mL for taleranol, zearalane, and zeranol and 100 μg/mL for DES and D_8DES).

ii. Working solutions: Dispense 200 μL of taleranol and 100 μL of zearalane, zeranol, DES, and D_8DES stock solutions into separate 100 mL volumetric flasks. Dilute to volume with methanol (concentration 0.4 ng/μL for taleranol, 0.2 ng/μL for zearalane and zeranol, and 0.1 ng/μL for DES and D_8DES).

b. Conjugated DES.

i. Stock solution: Take 1 ampoule of DES-MG (equivalent to 1 mg free DES) and dissolve the contents with methanol in a 100 mL volumetric flask (concentration equivalent to 10 μg/mL free DES).

ii. Working solution: Dispense 1 mL of stock solution into a 100 mL volumetric flask. Dilute to volume with methanol (concentration equivalent to 0.1 ng/μL free DES).

NOTE: Allow standards to equilibrate to room temperature before use.

3. Storage Conditions

All standards should be kept frozen at 0° C or lower if not in use.

4. Shelf Life Stability

a. Stock solutions: One year.

b. Working standards: Up to 6 months.

DETERMINATIVE/CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

NOTE: All tissues should be kept frozen at all times until assayed.

CAUTION: Usual glassware washing procedures are not sufficient for removal of residue from the 50 mL Pyrex centrifuge tubes. It is recommended that all such tubes be boiled for 2 hours in Decon solution prior to conventional cleaning. This boiling step may be eliminated if tubes are cleaned immediately after use.

a. Weigh 5.0 g + 0.1 g blended liver or ground, homogenized muscle into 50 mL polypropylene centrifuge tube.

b. Fortify each sample and control sample with 50 μL (concentration 0.2 ng/μL) of zearalane and 50 μL (concentration 0.1 ng/μL) of D_8DES internal standard (equivalent to 2.0 and 1.0 ppb) respectively.

c. For fortification curve for linear regression analysis, add zeranol (concentration 0.2 ng/μL), taleranol (concentration 0.4 ng/μL), and DES-MG (concentration 0.1 ng/μL) as follows:

Concentration			*μL of Spike Solution Added*
DES	*Zeranol*	*Taleranol*	
0.00 ppb	0.00 ppb	0 ppb	0.0 μL
0.50 ppb	1.00 ppb	2 ppb	25.0 μL
1.00 ppb	2.00 ppb	4 ppb	50.0 μL
2.00 ppb	4.00 ppb	8 ppb	100.0 μL

If DES is suspected at 0.25-0.5 ppb, spike at a lower level.

d. With repipet, add 11 mL of 0.04M sodium acetate buffer.

e. Homogenize for 1 minute, using Tissuemizer.

f. Adjust pH to 4.25-4.75 with glacial acetic acid. Add 5 drops of glacial acetic acid with a Pasteur pipet, vortex mix, and check pH (5-8 drops are usually required).

g. Add 100 μL β-glucuronidase to each sample (approximately 10,000 units). Cap tube and vortex mix thoroughly for about 10 seconds.

h. Incubate overnight (16-18 hours) at 37° C or place overnight in a constant temperature water bath set at 35-37° C.

i. After incubation, add 16.0 mL acetonitrile to each tube, using a repipet, and shake moderately by hand or in a Rotorack or Eberbach shaker for about 5 minutes.

j. Centrifuge samples at a minimum G force of 2450 for 5 minutes.

NOTE: g force = (1.118×10^{-5})(radius of rotation in cm)$(RPM)^2$

radius of rotation = distance from center shaft to the furthest inside tip of the tube.

DETERMINATIVE/CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE (Continued)

k. Decant the supernatant into a 50 mL screw-cap glass-stoppered standard taper centrifuge tube.

NOTE: If a small amount of floating debris is transferred with the supernatant, it will not interfere with the test.

l. Add 2 mL dichloromethane and 8 mL hexane to the supernatant.

m. Shake moderately by hand for 1 minute (or use Rotorack or Eberbach shaker).

n. Centrifuge at a minimum G force of 1060 for 2 minutes.

o. Transfer middle layer (acetonitrile) to a clean 20 mL scintillation vial, using a 10 mL disposable pipet, *without* removing any of the other layers.

p. With repipet, add another 4 mL of acetonitrile to the remaining two layers in the 50 mL centrifuge tube.

q. Shake moderately by hand for 1 minute (or use Rotorack or Eberbach shaker).

r. Centrifuge at a minimum G force of 1060 for 2 minutes.

s. Repeat step o above, recovering as much of the middle acetonitrile layer as possible, using a 10 mL disposable pipet, *without* removing any of the other layers.

Stop point: Samples may be stored for up to 1 hour at room temperature at this point.

t. Gently evaporate the combined acetonitrile layers to dryness with filtered house or compressed air at 60° C, using the Fisher Model 190 sample concentrator, or a gentle stream of nitrogen, using the custom-drilled heating block set at 60° C.

NOTE: All acetonitrile must be removed. This takes 45-60 minutes.

u. With repipet, add 2 mL 1:1 isopropanol:methanol and dissolve residue. Let set 5 minutes and vortex until dissolved.

v. With disposable pipet, add 1.5 mL 2N sodium hydroxide solution and vortex mix or swirl for 5-10 seconds.

NOTE: Column cleanup *must* proceed at this point.

w. Take apart TYPE AS cartridges; decant liquid above the ion exchange bed. Place the cartridges on the inner ring of the rotor. Place the plastic waste cups and plastic sample recovery cups on the outside ring of the rotor.

x. Load sample solutions from step v above onto the cartridge bed. Rinse the vials with 1.0 mL of isopropanol:methanol and also add this to the cartridges.

DETERMINATIVE/CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE (Continued)

y. Close the lid of the Prep I and select Program 7. Advance the program to step 2 with the STEP ADVANCE switch and start the processor with the STEP ACTIVATE/HOLD switch. Allow the unit to run for 4 minutes and then stop it with the STEP ACTIVATE/HOLD switch. Open the lid and empty the waste cups and return them to the outer rotor.

z. With repipet, add 4 mL methanol to each cartridge and repeat step y above.

aa. Add the following solvents to solvent reservoirs 1, 2, 3, and 4 respectively.

i. Wash solvent 1: Place 18 mL of distilled deionized water in reservoir 1. This is equivalent to 1.4 mL per cartridge.

ii. Wash solvent 2: Place 33 mL of 5% aqueous acetic acid in reservoir 2. This is equivalent to 2.75 mL per cartridge.

iii. Wash solvent 3: Place 22 mL of 25% aqueous methanol in reservoir 3. This is equivalent to 1.75 mL per cartridge.

iv. Elution solvent: Place 33 mL ± 3 mL of 100% methanol in reservoir 4. This is equivalent to 2.75 mL ± 0.25 mL per cartridge.

NOTE: The volume of methanol elution solvent may require adjustment (± 3 mL) such that the final volume in step cc below is < 0.5 mL. The specific volume required for each Prep I unit in each laboratory can be predetermined by the analyst.

bb. Select Program 7 and advance to step 3 with the STEP ADVANCE switch. Set the evaporation temperature to 55° C (approximately setting 5). Start the program with the START switch.

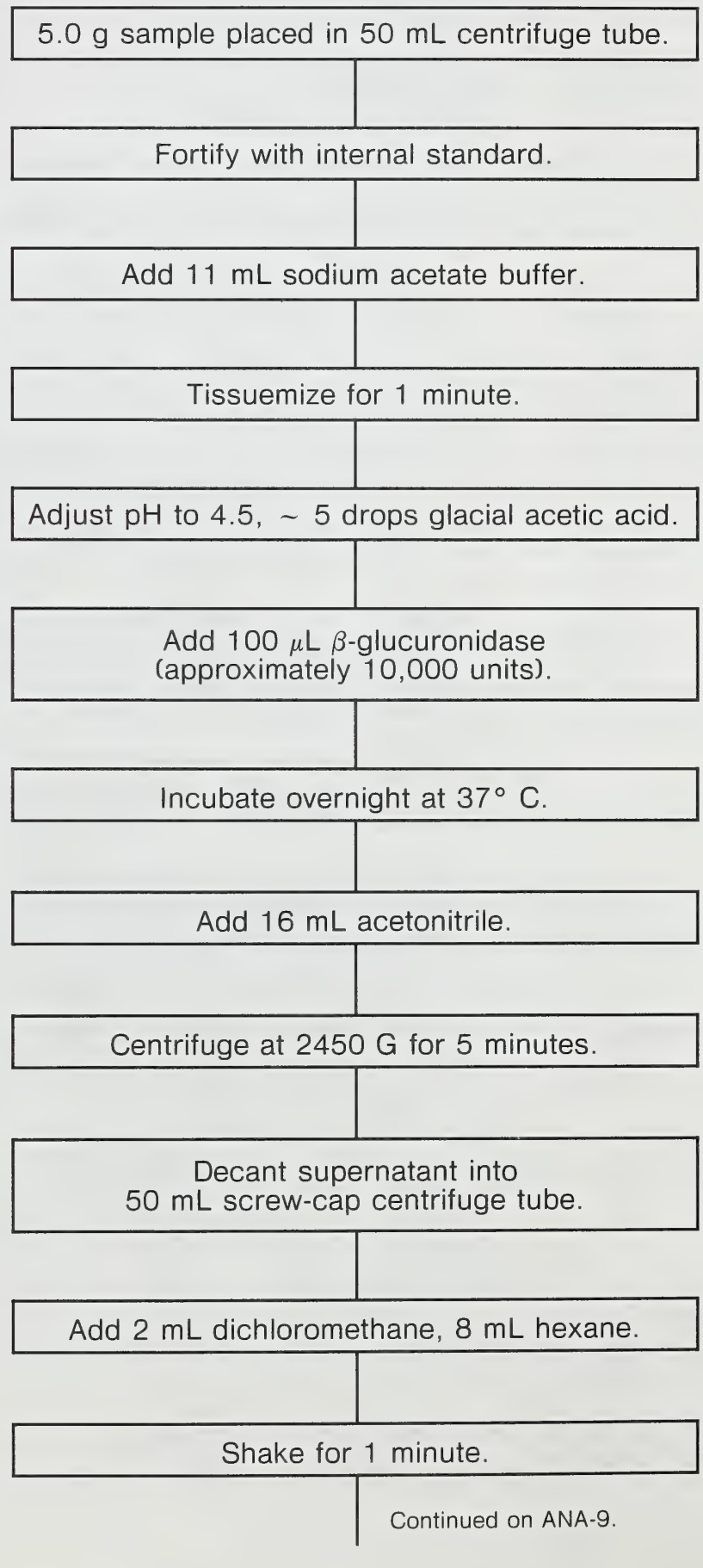
cc. After the program is finished, remove the plastic recovery cups and transfer methanol eluate (0.1-0.5 mL) with a Pasteur pipet to a tapered-bottom 1 mL auto sampler vial. If a sample volume exceeds 0.5 mL, part of the volume may be transferred and partially evaporated in the custom-drilled heating block before transferring the final portion. Rinse the recovery cup with 100 μL of methanol, using an Eppendorf pipet, and transfer to the sampler vial. Evaporate samples just to dryness under low nitrogen flow in a custom-drilled heat block set at 60° C. The sample is ready for GC/MS analysis.

NOTE: Extracts must be stored in a freezer at 0° C or less, and should be analyzed within 2 days. Midwestern Laboratory experience indicates, however, that extracts may be stored frozen up to a week without significant loss.

DETERMINATIVE/CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE (Continued)

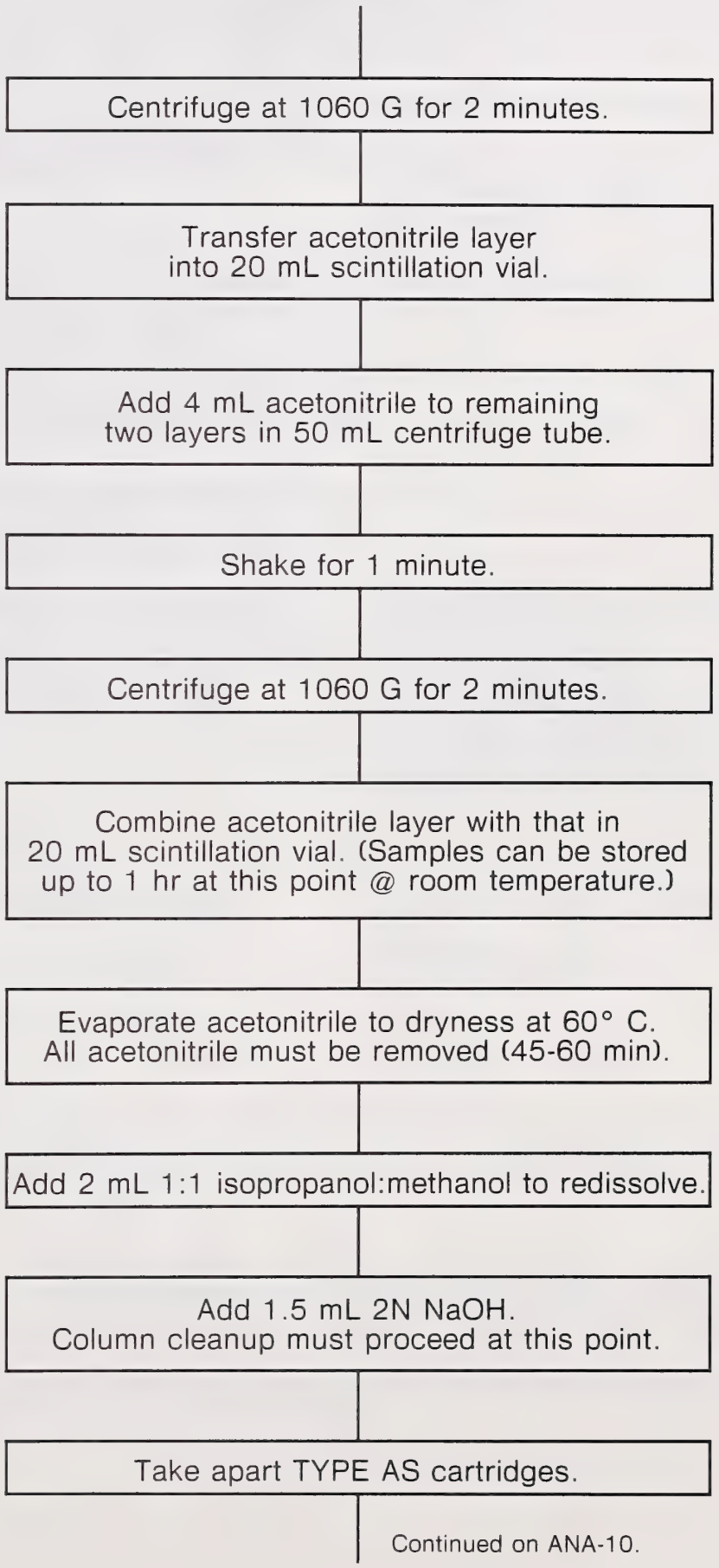
2. Flow Chart Summary



Continued on ANA-9.

DETERMINATIVE/CONFIRMATORY METHOD

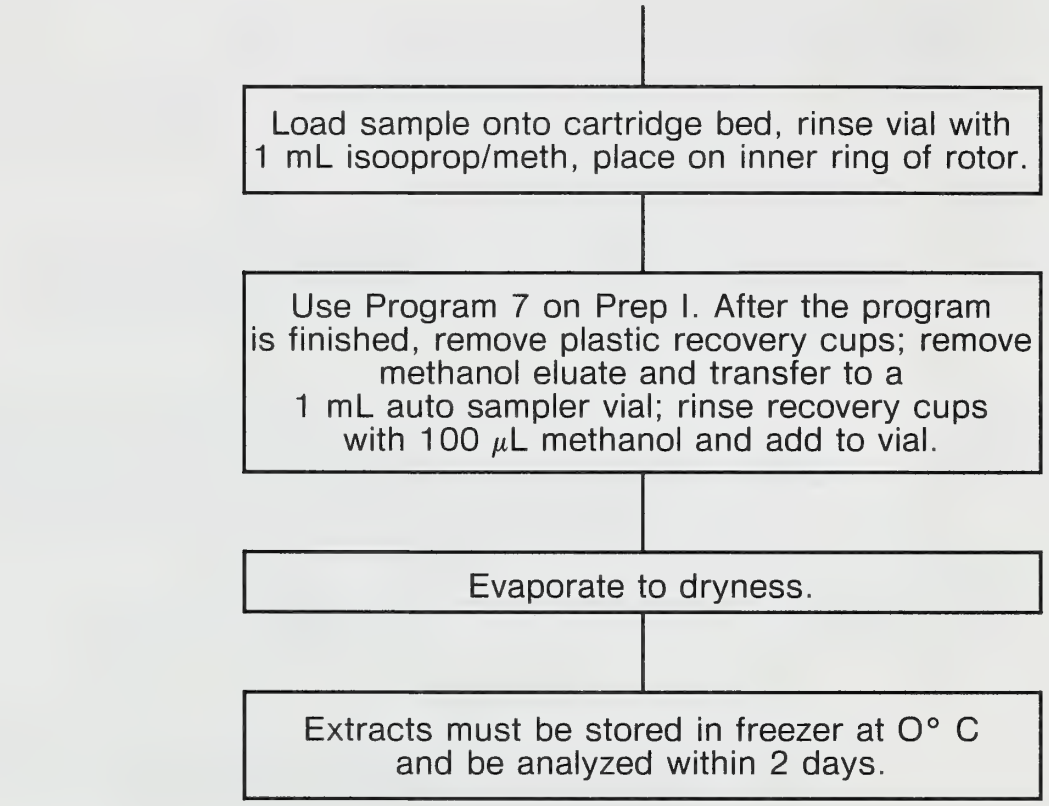
E. EXTRACTION PROCEDURE (Continued)



Continued on ANA-10.

DETERMINATIVE/CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE (Continued)



DETERMINATIVE/CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. GC parameters. Optimize instrumental conditions to obtain maximum sensitivity and adequate baseline resolution of peaks of interest, especially zeranol. The following table represents *one* optimization possibility.

i. GC	Hewlett Packard 5890
ii. Column	Crosslinked 100% methylsilicone HP special purpose 12 m, 0.2 mm i.d., 0.33 micron film thickness (16 m, 0.20 mm i.d., 0.5 micron film thickness has been used successfully)
iii. Injector	Heated splitless, 260° C minimum
iv. Linear carrier gas flow	30 cm/sec, helium
v. Source temperature	250° C
vi. Interface temperature	260° C
vii. Temperature program	Hold at 130° C for 1 minute; 20° C/min to 230° C; 5° C/min to 295° C. (If lower final temperature of 285° C is desired, sufficient time between injections must be given to elute several possible interfering peaks.)

b. MS parameters.

i. Mass spectrometer	Hewlett Packard 5970A-MSD
ii. Ionization mode	Electron impact
iii. Electron energy	70 eV
iv. Calibration	Autotune

2. General Operation

a. Reconstitute the dry residue with 10 μL of ethyl acetate and derivatize the samples with BSTFA plus 2% TMSI catalyst using an on-column derivatization technique.

b. Draw 1.0-2.0 μL of sample into the syringe, followed by a 1 μL air space and 2.3 μL of derivatization agent. (Wipe syringe needle with Kimwipe soaked with ethyl acetate before inserting into the derivatization agent.)

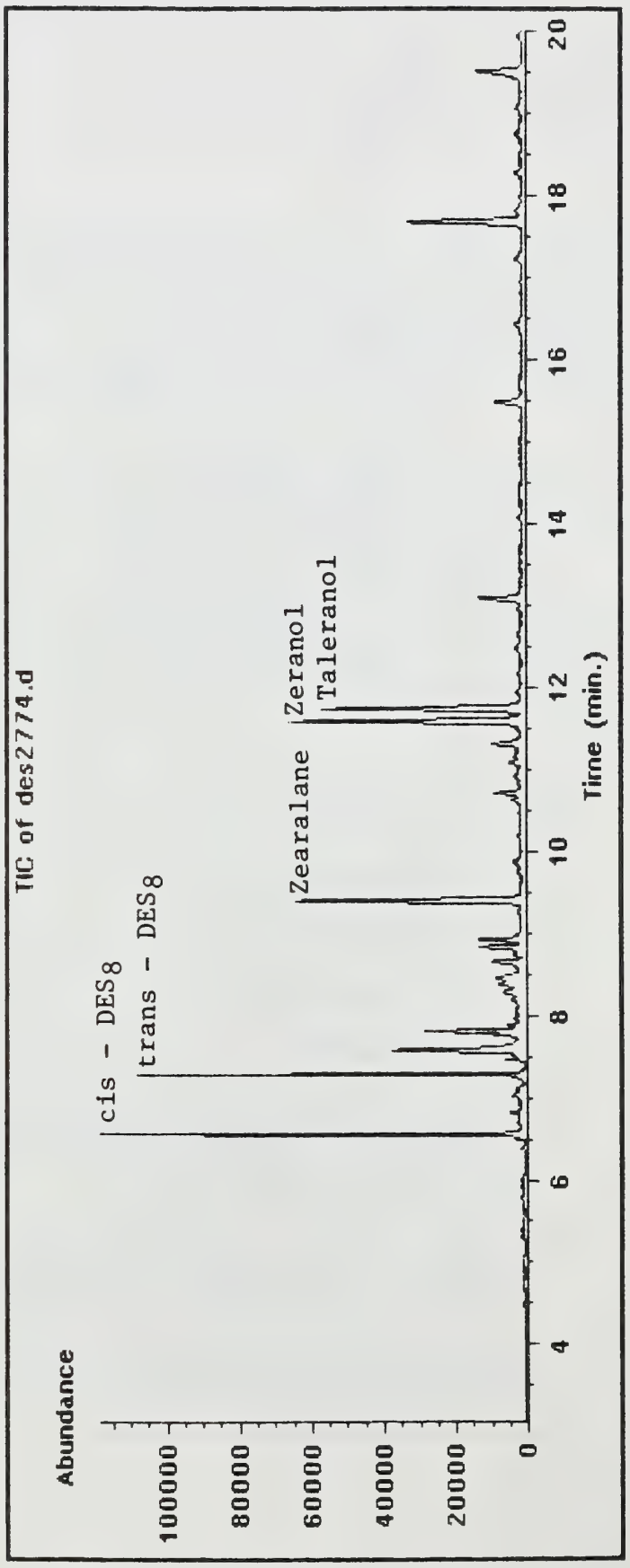
c. Inject the entire contents (3-5 μL total) into the heated injection part of the GC operating in the splitless mode.

DETERMINATIVE/CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. Sample Chromatogram

MASS SPECTRUM OF ZERANOL-INCURRED LIVER TISSUE



DES-d_8 = 1.0 ppb
Zearalane = 1.0 ppb

DETERMINATIVE/CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION (Continued)

4. Retention Times

Approximate retention times (min) with above conditions:

a. cis DES—7.3.

b. trans DES—8.0.

c. Zearalane—10.3.

d. Zeranol—12.6.

e. Taleranol—12.8.

5. Ions Monitored

Analyte	*Ions (Dwells = 100 milliseconds)*
a. Zearalane	435
b. Zeranol	538, 523, 433, 379
c. D_8DES	420
d. DES	412, 397, 383
e. Taleranol	538, 523, 433, 379

6. References

a. Determination of Diethylstilbestrol and Zeranol in Bovine Liver, Extraction, Screening, Quantitation, and Confirmation. Prepared by Covey, Stilvestre, and Henion, Cornell University, Ithaca, NY for USDA, October 15, 1985.

b. Quantitation of Zeranol and Diethylstilbestrol in Beef Liver, Kidney, and Muscle by GC-MS. Agriculture Canada, Animal Pathology Laboratory, Saskatoon, Saskatchewan, HOR-DP01.TXT—September 1986.

c. Method Extension Study on Ovine Tissue for Taleranol, DES, and Zeranol. September, 1988.

DETERMINATIVE/CONFIRMATORY METHOD

G. CALCULATIONS

1. Procedure

a. Read tangential area counts or peak height for the selected ions from the integration report and tabulate.

b. Obtain calibration curve values by ratioing the ion area of the analyte in question to the appropriate internal standard, as below:

$$\text{zeranol} = \frac{\text{Area counts or peak height for ions 538 + 523 + 433}}{\text{Area counts or peak height for ion 435}}$$

$$\text{taleranol} = \frac{\text{Area counts or peak height for ions 538 + 523 + 433}}{\text{Area counts or peak height for ion 435}}$$

$$\text{DES} = \frac{\text{Area counts or peak height for ion 412, cis + trans}}{\text{Area counts of peak height for ion 420, cis + trans}}$$

c. Using linear regression, construct a standard curve of DES or zeranol ion ratios vs concentration of DES or zeranol.

The equation: $y = mx + b$.

where: y = ion ratio

x = conc. in ppb (μg/kg)

m = slope

b = y intercept

The correlation coefficient should be > 0.995.

d. From the ion ratios, using the regression slope and intercept, calculate the concentration for each sample.

2. Criteria for Confirmation

a. Suspect positive samples will be reanalyzed in duplicate, the set to include a tissue blank and three fortifications to bracket the estimated level of the suspect positive.

b. All monitored ions must be present for all isomers.

c. Blank tissue must show no interferences.

d. Ion ratios must agree within 30% of those of the fortified standard.

Compounds	*Ion Ratios*
Zeranol, Taleranol	523/538 433/538 379/538
DES (cis + trans)	383/412 397/412

e. Retention time must agree within 0.3 min of that for the fortification extract.

DETERMINATIVE/CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination and Confirmation of Diethylstilbestrol/Zeranol/Taleranol in Bovine and Ovine Tissues

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Acetonitrile, hexane, dichloromethane, ethyl acetate.	These solvents are corrosive and flammable. Vapors can cause severe skin, eye, and respiratory irritation.	Work should be performed in an efficient fume hood away from heat-generating devices.
Sodium hydroxide	Caustic; may cause irreversible skin and eye damage.	Wear the required protective equipment. Avoid spillage or spattering.

4. Disposal Procedures

Acids and bases	See above	Dilute and flush into waste sink with large amounts of water.
Organic solvent mixtures	See above	Segregate chlorinated from nonchlorinated solvents until disposal by contract or in-house specialist.

DETERMINATIVE/CONFIRMATORY METHOD

I. WORKSHEET

The worksheet on the facing page, *DES/Zeranol/Taleranol*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: DES/ZERANOL/TALERANOL

Analyst: _____ **Date Started:** _____ **Date Completed:** _____ **Matrix:** _____

DES Standard Curve Data

Standard	ppb	Ratio 412/420
1		
2		
3		
4		

Linear Regression

b =
m =
r =
s =

Zeranol Standard Curve Data

Standard	ppb	Ratio 433 + 523 + 530/435
1		
2		
3		
4		

Linear Regression

b =
m =
r =
s =

Taleranol Standard Curve Data

Standard	ppb	Ratio 433 + 523 + 530/435
1		
2		
3		
4		

Linear Regression

b =
m =
r =
s =

	Laboratory Number	Control Number	Sample Weight 5 g ± 0.1 g	pH 4.5 ± 0.25	DES Ratio	DES ppb	Zeranol/ Zeralane Ratio	Taleranol Present (y/n)	Zeranol ppb	Taleranol Zeralane Ratio	Taleranol ppb

DETERMINATIVE/CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound List	*Analytical Range (ppb)*	*Apparent Acceptable Recovery %*	*Repeatability %CV*
DES	≥ 0.25 ppb	80-100	≤ 20
Zeranol	≥ 0.50 ppb	80-100	≤ 20
Taleranol	≥ 2.0 ppb	40-60	≤ 20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample Weight	5.0 g ± 0.1 g
b. Hydrolysis pH	4.5 ± 0.25
c. β-Glucuronidase	100 μL
d. Incubation	35-37° C
e. Evaporation to dryness	Remove all acetonitrile
f. Sodium hydroxide	1.5 mL of 2N

3. Readiness To Perform

a. Familiarization.

i. Phase I: External standard curve is not applicable for this method. Proceed to Phase II.

ii. Phase II: Analysts's self-fortified samples—Duplicate recovries at the fortified levels as listed below for three consecutive sets including:

(a) 1 set of liver fortified with DES and zeranol.

(b) 1 set of liver fortified with DES and taleranol.

(c) 1 set of muscle fortified with DES and zeranol.

DES (ppb)	*Zeranol (ppb)*	*Taleranol (ppb)*
0	0	0
0.5	1.0	2.0
1.0	2.0	4.0
2.0	4.0	8.0

iii. Phase III: Check samples for analyst accreditation:

(a) 15 liver samples, including:

(1) 5 samples fortified with DES and taleranol.

(2) 10 samples fortified with DES and zeranol.

(b) 10 muscle samples fortified with DES and zeranol.

DETERMINATIVE/CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

(c) Report analytical findings to Chemistry Division, QSB.

Notification from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Monthly by each analyst.

ii. Records to be maintained by analyst and reviewed by supervisor and/or Laboratory QA Officer for:

(a) All replicate findings.

(b) Appropriate CUSUM charts.

(c) Coefficient of correlation of all internal standard curves.

b. Acceptability criteria.

Refer to section J.1 above.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver or muscle.

b. Sample receipt size: Varied; enough to obtain matrix required for all quantitative and confirmation tests and reserve sample.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: ≤ 6 months.

ii. Condition: Frozen.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	a. 12 units as determined by DuPont Prep I apparatus. b. Screen: Set will include 1 fortification at action level and 11 samples. c. Quantitation: Set will include a 4-point fortification curve (as described in section E.1.f.) and up to 4 samples in duplicate.

APRAMYCIN

Contents

Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 9
G. [Reserved]
H. [Reserved]
I. [Reserved]
J. Quality Assurance Plan 10

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Samples are treated with potassium hydroxide and heated. After cooling, the samples are blended with trichloroacetic acid, the precipitate is removed by centrifugation, and the supernate is passed over an ion exchange column. Subsequently the column is washed and the apramycin is eluted with ammonium hydroxide. The samples are taken to dryness and then reconstituted in a small amount of water. An aliquot is spotted on a silica gel plate, developed, and bioautographed using *Bacillus subtilis* as the test organism.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

a. Meat grinder: Hamilton Beach, Model 223, or commercial-type grinder.

b. Tissue Blender: Waring-type blender such as Hamilton Beach, Model 266, equipped with blender heads to fit a one-half pint Mason jar, or Tissuemizer, homogenizer, Tekmar Co., or equivalent.

c. Centrifuge: International centrifuge Model V, size 2, with either a No. 239 or No. 267 horizontal head (equipped for 250 mL glass bottles), or equivalent.

d. Sample heating equipment: Steam chest at atmospheric pressure, autoclave, or boiling water bath.

e. Hot plate: Corning PC 100 or equivalent, or steam table.

f. Chromatography columns: 10 mm id × 250 mm (with 100-250 mL reservoir suggested) and Teflon stopcocks.

g. Hot water bath.

h. Incubator.

i. Colorimeter: Bausch and Lomb spectronic-20 or equivalent.

j. Thin-layer chromatography developing chambers.

k. Photographic equipment: Polaroid Model MP3, or equivalent.

l. Schleicher and Schuell No. 588 filter paper, or equivalent.

m. Chromatography spray flask.

n. Artist's atomizer-type sprayer such as Grumbacher atomizer No. 836.

o. Vortex mixer such as Vortex Genie, Scientific Industries, Inc.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Potassium hydroxide A. R., 0.1 N, 0.05 N.

b. Trichloroacetic acid A. R., 25% w/v.

c. Sodium hydroxide A. R., 50% w/v.

d. Ammonium hydroxide A. R., 1.0 N.

e. Methanol A. R.

f. Chloroform A. R.

g. Amberlit CG 50 Li+ cycle: Slurry new CG-50 resin (Amberlite CG-50 AR, 100-200 Mesh) in 1.0N H_2SO_4 for a period of three hours. Wash the resin with distilled water until the pH of the wash water is above 5.0. Add LiOH slowly, stirring, until the pH of the slurry remains between 7.0 and 8.0. Allow the slurry to stand overnight. Wash the resin with distilled water at least ten times. Adjust the pH of the resin-water slurry to 7.0 with 1.0N H_3PO_4. Store the slurry in tightly sealed glass jars until ready for use.

h. Thin-layer chromatographic plates: Woelm Silica Gel G 250 micron precoated TLC plates from Analtech, Inc., or equivalent.

i. Glass wool.

j. Agar Medium No. 23 N/1: Dissolve 0.69 g K_2HPO_4; 0.45 g KH_2PO_4; 2.5 g yeast extract (Difco); 10.0 g glucose (cerelose); and 15.0 g Noble Agar (Difco) in deionized water to give 1 L total volume. Autoclave the solution for 15-20 minutes at 121° C. Adjust the pH to 6.0 just before use.

k. Agar Medium No. 23 N/2: Dissolve 0.69 g K_2HPO_4; 0.45 g KH_2PO_4; 2.5 g yeast extract (Difco); 10.0 g glucose (cerelose); and 6.0 g Noble Agar (Difco) in deionized water to give 1 L total volume. Autoclave the solution for 15-20 minutes at 121° C. Adjust the pH to 6.0 just before use.

l. *Bacillus subtilis* stock suspension.

   i. Wash the growth of *Bacillus subtilis,* American Type culture Collection No. 6633, from one penassay seed agar slant (culture medium No. 1 from Grove and Randall, Assay Methods of Antibiotics) with 3 to 5 mL of sterile deionzied water onto the surface of a Roux bottle containing 300 mL of penassay seed agar with 0.03% manganous sulfate added.

   ii. Incubate the culture for one week at 37° C.

   iii. Following incubation, wash the growth from the agar surface of the Roux bottle with approximately 50 mL of sterile deionized water.

   iv. Transfer the wash to a sterile 250 mL centrifuge bottle and hold the organisms at 65° C in a water bath for 30 min.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

v. Centrifuge the suspension and discard the supernatant liquid.

vi. Repeat the resuspension and washing of the organisms three times.

vii. After the final wash, heat shock the cells again by immersion in the 65° C water bath for 30 min and suspend them in 30 mL of sterile deionized water.

NOTE: This stock suspension is stored at 4° C to 5° C for use.

m. *Bacillus subtilis* inoculum: Prepare the inoculum by diluting the stock suspension with sterile deionized water to obtain a 20% light transmittance at 530 nm using a Spectronic-20 colorimeter. This suspension may be used for 1 week when stored under refrigeration.

n. A 2 mg/mL solution of INT [2(p-iodophenyl)-3-p-nitrophenyl-5-phenyl tetrazolium chloride]. Dissolve the INT in 10% of final volume of methanol: deionized water.

o. Developing solution for TLC: methanol:chloroform:concentrated ammonium hydroxide (55:15:50).

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

Standard solutions.

a. Weigh a quantity of standard apramycin base to contain 100 mg of apramycin activity.

b. Quantitatively transfer the weighed standard to a 100 mL volumetric flask and dissolve in deionized water. Dilute to the mark with water and mix thoroughly. This standard solution contains 1,000 μg/mL of apramycin activity.

c. Pipette 1 mL of 1,000 μg/mL standard into a 100 mL volumetric flask with a volumetric pipette. Add a few drops of concentrated ammonium hydroxide and dilute to the mark with water and mix thoroughly. This standard contains 10 μg/mL of apramycin activity.

d. Pipette 5 mL of the 10 μg/mL standard solution into 40 mL volumetric flask and make to volume with deionized water. This standard contains 1.25 μg/mL of apramycin activity.

2. Storage Conditions

Standard solutions should be stored lightly stoppered at 4° C.

3. Shelf Life Stability

Standard solutions are stable at least 30 days when stored under refrigeration as described in section 2.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction and Cleanup

a. Weigh a representative, previously ground tissue sample (25 g) into a suitable glass container compatible with the type of blender to be used.

NOTE: Although Pyrex containers are preferred, one-half pint Mason jars may be used when only Waring-type blenders are available.

b. For lean, kidney, fat, and skin tissues, add 4 mL of 0.1N KOH per gram of tissue. For liver tissue samples, add 4 mL of 0.05N KOH per gram.

c. Blend until uniform and rinse blender head into the sample container with deionzied water.

d. Cover each sample container with commercial-grade aluminum foil.

e. Heat the samples to >85° C for at least 20 min in a steam chest, autoclave, or boiling water bath.

f. Punch a small hole in the aluminum foil and cool the samples to near room temperature in a refrigerator (30-40 min) or by partial immersion of the sample containers in warm and then cold running tap water.

g. Add 1 mL of 25% trichloroacetic acid per g of tissue.

h. Blend until slurry is uniform.

i. Transfer the slurry to a 250 mL centrifuge bottle.

j. Centrifuge 15 minutes at 1700-1800 rpm.

k. Filter supernate through Schleicher and Schuell (S and S) No. 588 filter paper, or equivalent, into a 250 or 300 mL Erlenmyer flask.

l. Adjust the filtrate pH to 7.0 ± 0.2 using 50% NaOH and 10N HCl.

NOTE: Be careful not to let the pH go above 8.0.

m. Prepare a CG-50 (Li+) column for each test and recovery sample processed.

   i. Place approximately 10 mL of deionized water into a 10 × 250 mm glass reservoir column. Insert a glass wool pledget and tamp it with a glass rod to eliminate air bubbles.

   ii. Add CG-50 (Li+) resin slurry to the column and drain it to the top of the resin. Discard all effluent.

n. Filter the pH 7.0 extract from step l through S and S No. 588 filter paper, or equivalent, into the prepared column.

o. Adjust flow rate to approximately 5 mL/min. Drain to top of resin.

p. Wash the filter and column with 100 mL boiling deionized water and drain to the top of the resin. Discard all column effluents up to this point.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

q. Place a 50 mL beaker in position to receive the column eluate.

r. Gently and carefully elute the column with 20 mL of 1N NH_4OH at the rate of approximately 3 mL per minute. Rinse the column with an additional 5 mL of NH_4OH and retain the combined eluates.

s. Evaporate the combined column eluates to 0.5-1 mL by adding 2 or 3 small carborundum boiling chips and either gently boiling on a hot plate or heating on a steam bath with a gentle stream of clean, dry air or nitrogen.

t. Quantitatively transfer the residue to a small sample vial or 15 mL conical test tube with the aid of three 1 mL rinses with 1N NH_4OH.

u. Evaporate the sample to dryness by heating on a steam bath or warm water bath and directing a continous stream of clean, dry air or nitrogen over the sample surface.

v. Reconstitute the sample to 0.10 mL with deionized water.

2. Preparation of Standard Recoveries

NOTE: Prepare control samples and standard recoveries with each set of experimental samples.

a. Weigh 25 g of the appropriate control tissue into blender jars.

b. Add the standard solution of apramycin (1.2 μg/mL in water) by pipette to give the desired concentration in the recovery samples. One negative control (no apramycin added) and three recovery samples containing 0.05, 0.1, and 0.2 μg apramycin per gram of tissue (1, 2, and 4 mL of the 1.25 μg/mL standard) respectively are to be processed with each set of experimental samples. The standard solution may be stirred into the tissue with a stainless steel spatula and rinsed with the added extraction solution.

c. Control and recovery samples are extracted as described for the experimental samples.

3. Thin-Layer Chromatography

a. Spot 20 microliters of the sample on a silica gel thin-layer chromatographic plate. A stream of warmed air may be used to facilitate spotting. Keep spot size $<$ 1.0 cm in diameter.

b. Develop the thin-layer chromatographic plate in a saturated tank using the developer of methanol:chloroform:concentrated ammonium hydroxide (55:15:50). Development time, approximately 2 hours.

c. Subsequent to development, allow the thin-layer plate to air dry thoroughly.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Preparation of Bioautographs

NOTE: Prepare the bioautographs essentially according to the method of Kline and Golab, *J. Chromatog.* 18, 409 (1965).

a. Melt agar media (refer to section C, Reagent and Solution List, items j and k) in a steam bath or autoclave.

b. Fix the thin-layer plate from section E.3.c above by spraying the surface with Medium No. 1 (refer to Section C, Reagent and Solution List, item j), using an artists' atomizer-type sprayer attached to the laboratory's compressed air supply. Spray until the entire plate is well coated (approximately 20 mL).

c. Place the TLC plate in a plexiglass holder (or other suitable container that will prevent contamination of the plate and drying of the agar during incubation).

d. Cool 50 mL of melted Medium No. 2 (refer to Section C, Reagent and Solution List, item k) to 52° C in a 125 mL Erlenmeyer flask and inoculate the agar with 0.1% (1 drop of *Bacillus subtilis*) inoculum suspension from section C, Reagent and Solution List, item m. Mix quickly by swirling.

e. Pour this seeded agar over the surface of the TLC plate.

NOTE: Pouring of the seed agar must be done rapidly and carefully to ensure an even agar overlay before the agar solidifies.

f. Allow the plate to cool until the agar sets. Leave the cover of the plate holder off to permit moisture to escape for a period of approximately 1 hr.

g. Place the cover on the plate holder and incubate at 30° C overnight.

h. Subsequent to incubation, spray the plate with a solution of INT (refer to section C, Reagent and Solution List, item n. Allow the color to develop for a period of approximately 1-2 hr. Additional sprayings (1 or 2) applied subsequent to the initial spraying's absorbtion into the agar are helpful in increasing the rate and degree of color contrast.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Estimation of Concentration

a. After incubation, locate the apramycin zones on the bioautograph at the R_f of approximately 0.55.

b. Estimate apramycin concentration by visually comparing zone sizes from experimental samples to zone sizes from standard recovery samples.

   i. Negative samples are reported as no activity at sensitivity of the lowest positive standard recovery.

   ii. Positive samples are reported as the concentration of the corresponding standard recovery level or as a range between two standard recoveries.

   NOTE: Apramycin levels obtained in this manner are regarded as approximate rather than absolute values.

c. If permanent records of bioautographs are desired, label the TLC plates for identification and photograph by reflected light.

NOTE: Polaroid photography of bioautographs has the advantage of producing a print immediately so that a satisfactory print may be obtained before destruction of the bioautograph.

d. Test sensitivity when standard apramycin is applied to the TLC plates in water solution is approximately 0.1 μg.

e. The lower limit of test sensitivity for standard recoveries processed by the extraction procedures described is approximately 50 ng of apramycin per gram of fresh tissue (50 ppb).

f. The assay is to be regarded as semiquantitative.

2. Reference

NADA 106-964, Eli Lilly.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Analyte	*Analytical Range (ppb)*	*Acceptable Recovery (%)*	*Repeatability %CV*
Apramycin	≥ 0.05	60-115	≤ 20

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	pH of Amberlite CG-50 resin	7.0 ± 0.2
b.	pH of agar media	6.0 ± 0.2
c.	Transmittance of inoculum	20 ± 2% at 530 nm
d.	pH of filtrate	7.0 ± 0.2
e.	Flow rate	< 5 mL/min
f.	Elution rate	< 3 mL/min
g.	TLC spotting	20 μl; < 1.0 cm diameter spot
h.	Plate drying	No ammonia odor
i.	Inoculation temperature	> 52° C but < 65° C
j.	Incubation temperature	30° C ± 2° C

3. Readiness to Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each: Blank, 0.05 ppm, 0.10 ppm, and 0.20 ppm.

ii. Phase II: Fortified samples—3 levels plus blanks over a period of three days, 12 total analyses: Blank, 0.05 ppm, 0.10 ppm, and 0.20 ppm.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III—Check samples for analyst accreditation. Fourteen samples from FSIS Western Laboratory. Samples submitted by supervisor if only one laboratory is performing this test. Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptable criteria

Refer to section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Minimum of 1 per week per analyst, or 20% of official samples analyzed whichever is smaller.

ii. Blind samples—require ''dummy forms.''

iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All percent recoveries.

(d) For all recoveries: the running average, standard deviation, and coefficient of variation of the last 10 acceptable recoveries.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

(a) Stop all official analyses for that analyst.

(b) Investigate and identify probable cause.

(c) Take corrective action.

(d) Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Swine kidney and muscle.

b. Sample receipt size, minimum: Varied; enough to obtain matrix for all analyses required.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: 90 days.

ii. Condition: Frozen.

6. Sensitivity

a. Lowest detectable level (LDL): 0.05 ppm.

b. Lowest reliable quantitation (LRQ): 0.05 ppm.

c. Minimum proficiency level (MPL): 0.1 ppm.

ARSENIC

Contents

Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. (Reserved)
G. Calculations 7
H. Hazard Analysis 9
I. Worksheet 10
J. Quality Assurance Plan 13

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The sample is ashed at 600° C and dissolved in dilute hydrochloric acid. Zinc is added; the arsenic is distilled as arsine (AsH_3) and collected in an iodine solution. Ammonium molybdate is added to the solution forming a heteropoly molybdiarsenate, which upon the application of heat, is reduced by hydrazine sulfate to form the ''molybdenum blue'' complex. The developed color is read on a suitable spectrophotometer at 840 nm.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Distillation apparatus—Kingsley-Schaffert distilling apparatus (Corning Glass Works, No. 33680) consisting of 125 mL flask, funnel trap, and bent dispersion tube.

b. Pipettes—various sizes.

c. Matched set of cuvettes approximately 19 × 105 mm.

NOTE: Glassware should not be subjected to routine washing with soap or detergents, which are often the source of phosphorous contamination. When soap or detergent is used, clean with aqua regia before use. Rinse delivery tubes by holding in slanted position with crook up and squirting jet of H_2O up and over inside crook until tube is filled; then rinse outside while tube drains. Repeat rinse three times. Rinse funnels in each direction alternately by filling end that is up and placing funnel on 1-hole rubber stopper in mouth of vacuum flask to pull H_2O through frit by vacuum.

2. Instrumentation

Spectrophotometer suitable for reading at 840 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Tissue solvent: $CHCl_3$ (or benzene)-acetone-absolute alcohol (1 + 1 + 2).

b. Dilute hydrochloric acid: Dilute 372 mL conc HCl to 1000 mL with distilled water (4.5N).

c. Potassium iodide solution 15%: Keep in dark. Discard when solution turns yellow.

d. Stannous chloride solution: 40 percent in diluted HCl (refer to item b above). Store in contact with metallic tin.

e. Zinc: Shot of uniform size and shape, ca. 0.5 g each. Prewash the zinc shots with petroleum ether to remove coating; remove solvent by evaporation on a steam bath and dry in an oven at 95° C.

f. Lead acetate solution: Prepare saturated aqueous $Pb(OAc)_2 \bullet 3H_2O$ solution in dropping bottle. Prepare fresh weekly or when solution becomes cloudy.

g. Iodine solutions:

   i. 0.02N: Dissolve 8 g KI and 2.54 g I_2 in small amount H_2O and dilute to 1 L with H_2O. Store in dark bottle.

   ii. 0.001N: Dilute 5 mL 0.02N I_2 to 100 mL with H_2O. Prepare fresh daily.

h. Ammonium molybdate solution: Dissolve 7.0 g $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$ in warm mixer of 70 mL H_2SO_4 and 300 mL H_2O, cool, and dilute to 500 mL with H_2O.

i. Hydrazine sulfate solution: Dissolve 0.3 g $N_2H_4 \bullet H_2SO_4$ in H_2O and dilute to 200 mL.

j. Absorbent cotton: metal-free.

k. Magnesium nitrate hexahydrate: $Mg(NO_3)_3 \bullet 6H_2O$, reagent grade. 50% solution (w/v)—Dilute 500 g $Mg(NO_3)_2 \bullet 6H_2O$ to 1 L with distilled water.

l. Dilute nitric acid (50%): One volume HNO_3 and one volume H_2O.

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

a. Inorganic arsenic standard solution.

19 μg/mL—Dilute 10.0 mL of a 1000 μg As/mL solution (Fisher, Cat. No. SO-A-449, or equivalent) to 100 mL with dilute hydrochloric acid (refer to section C, Reagent and Solution List, item b).

b. Organic arsenic standard solutions. (Arsenilic acid, Fisher, Cat. No. #1369, or equivalent).

i. 1 mg As/mL: Dissolve 0.2897 g arsenile acid (based on 100% purity) in water and dilute to 100 mL.

ii. Dilute 1.0 mL of solution b.i above to 100 mL with water (10 μg As/mL).

iii. Dilute 1.0 mL of solution b.i above to 200 mL with water (5 μg As/mL).

iv. Dilute 1.0 mL of solution b.i above to 500 mL with water (2 μg As/mL).

NOTE: Because the form of arsenic used in raising animals and poultry is organic, the use of organic arsenic standards is preferable.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

NOTE: A reagent blank must be analyzed with each set of samples, the absorbance of which should not exceed 0.15.

a. Sample preparation may be varied according to the type or quantity of sample available. Where sample quantity is ample and a portion representative of the whole sample is desired, pass the entire sample through the fine plate of a meat grinder at least twice (liver will only make one pass, or it may be blended instead of ground).

   i. Mix sample thoroughly and weigh desired amount (usually 10 g) into 50 mL Vycor crucible.

   ii. Add 8 mL magnesium nitrate 50% solution (4 g magnesium nitrate hexahydrate per 10 g of sample); mix thoroughly with clean plastic stirring rod.

   iii. Spread the mix in an even layer around the sides of the crucible.

b. When samples are small, the tissue solvent may be employed to assist in comminuting and mixing.

   i. Weigh a convenient amount of the sample into an homogenizer or blender vessel, add the proper amount of $Mg(NO_3)_2 \cdot 6H_2O$ (4 g per 10 g of sample), and add a convenient amount of the tissue solvent—usually 30 mL per 10 g sample.

   ii. Blend about 1 min and weigh into a Vycor crucible an amount that will contain the desired amount of sample.

   iii. The excess solvent and water (from tissue) must be carefully evaporated before the sample is burned. Remove the solvent by placing the crucible on a steam bath. After all the solvent has been evaporated, place the crucible in an oven at about 95° C to remove the water.

2. Determination

a. Place the crucible in a forced air oven. Dry at ca. 90° C overnight or at ca. 110° C for 4 hr. Place in cool muffle furnace that is set to gradually come to 600° C (this can be done overnight). When all visible carbon is burned, cool the crucibles, and cool the furnace.

b. Dampen the ash with 50% HNO_3. (Do not dissolve ash completely.) Place on low-heat hot plate to dry the ash.

c. Replace the crucibles in the cooled furnace (100° C) and heat gradually to 600° C. Hold at 600° C about 1 hr or until it is certain that all nitric acid fumes have been driven off. (If the furnace is small, those crucibles near the door may not be as hot as some others. In this case, their positions should be reversed. When this is necessary, two periods of about 40 min each are necessary.)

d. Remove crucibles from the furnace and allow to cool.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

e. Dissolve ash in 4.5N hydrochloric acid. Three 10 mL portions of acid (delivered with all-glass syringe without needle) are used to dissolve and transfer the ash to the 125 mL flask and a fourth 10 mL portion is used to wash down the sides of the flask.

f. Cool.

g. Add 2.0 mL of 15% potassium iodide solution and mix thoroughly by swirling flask.

h. Add 1.0 mL of 40% stannous chloride solution and mix thoroughly by swirling flask. Allow to stand in ice bath at least 15 min (but not more than 30 min). The 15 min waiting time is most convenient for making other preparations for the distillation. Wet all glassware and allow to drain for 30 min.

i. Place 7.0 mL of 0.001N iodine in each cuvette.

j. Place a small ball of absorbent cotton in top of each funnel and dampen with saturated lead acetate solution. Using distilled water as a lubricant, join the delivery tube firmly to the funnel.

k. Fill a 600 mL beaker with finely crushed ice layered between the levels of the cuvette holder. Add ice water to about ⅔ the height of the beaker and place the cuvettes in the holder. (It may be necessary to make a path through the ice for the cuvette).

l. At the end of 15 min, lubricate the lower joint of the funnel with distilled water, add approximately 12.5 g of zinc (previously weighed) to the flask, join the flask and funnel firmly, and place delivery tube in cuvette as quickly as possible. Allow distillation to continue 1 hr.

m. At the expiration of 1 hr, carefully and slowly remove the delivery tube from the cuvette, allowing liquid to drain as tube is being removed.

n. Add 0.5 mL ammonium molybdate solution to the cuvette and mix thoroughly.

o. Add 0.3 mL hydrazine sulfate solution to the cuvette and mix thoroughly.

p. Place cuvette (in the cuvette holder) in a boiling water or steam bath for 10 min.

q. Remove from the bath, wipe the cuvette dry with soft, lintless material and place in a cool, dark place for about 1 hr before reading.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

It is *necessary* that the standard curve be made from distilled arsenic and it is *advisable* that the added arsenic be carried through the entire procedure with an arsenic-free sample of tissue.

Prepare standard curve with 10 g samples of arsenic-free liver and 8 mL of 50% of magnesium nitrate. When using the standard inorganic arsenic solutions, 100 μg As/mL, add known amounts of arsenic with a 200 μL syringe to each in a definite progression of 50, 100, 150, 200, and 250 μL respectively equal to 5, 10, 20, and 25 run at one time. When using the standard organic (arsenilic acid) solutions, add known amounts of arsenic to each in a definite progression such as 0, 2, 4, 6, 8, 10, etc., micrograms of As or 0, 3, 6, 9, 12, 15, etc., micrograms of As. Repeat at least three times. Determine the mean for each level and prepare a fitted line by linear equation. The following suggested formula may be used:

$$Y = a + bX$$

The ''X'' values are the various levels of arsenic used (2, 4, 6, etc., micrograms) used in obtaining the data. The levels of ''X'' should be shown as micrograms per gram. The ''Y'' values are the means of the absorbance readings obtained at each level of arsenic. The ''a'' and ''b'' values are constants that are derived from the data.

Using the ''X'' and ''Y'' values to drive the ''a'' and ''b'' values and their subsequent use in the above equation is known as the ''method of least squares.''

A line plotted by this method will give the best fit for the data available. The ''a'' and ''b'' values are derived from the following equations:

$$b = \frac{\Sigma(XY) - \frac{\Sigma(X)\,\Sigma(Y)}{N}}{\Sigma(X^2) - \frac{\Sigma(X)^2}{N}}$$

$$a = \frac{\Sigma(Y)}{N} - \frac{b\,\Sigma(X)}{N}$$

N = the number of levels of X

Arsenic-free tissue (if not available, use previously analyzed tissue) with known amounts of standard arsenic added would be analyzed with actual samples to determine the efficiency of the system.

Calculations:

Arsenic (ppm) = A • B

$$A = \frac{10g}{\text{Sample wt(g)}}$$

B = microgram of arsenic per gram (standard curve)

NOTE: 10 g based on sample sized used to prepare standard curve.

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

2. Reference	*Official Methods of Analysis of the Association of Official Analytical Chemists,* 14th Edition.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Arsenic Analysis (Spectrophotometric)

2. Protective Equipment — Safety glasses, plastic gloves, heat resistant glves, and lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Tissue solvent: $CHCl_3$-acetone-absolute alcohol	This solvent is flammable and can irritatate the skin, eyes, and respiratory system.	Perform this step in a fume hood and insure that the solvent is completely removed from the sample before placing it in the oven.
D. Determination		
Dampen the ash with 50% HNO_3—etc.	Acid fumes and spattering, which can result in burns and severe skin, eye, and respiratory irritation.	Always perform this step in a fume hood.
Dissolve ash in 4.5N HCl, etc.	Same as above.	Same as above.

4. Disposal Procedures

Acidic solutions	Same as above.	Dilute and flush into an acid disposal sink located in a well-ventilated area.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Arsenic (Spectrophotometric) Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

Worksheet: Arsenic (Spectrophotometric) Analysis

Analyst: _____ **Date Started:** _____ **Date Finished:** _____

				Time Started	Time Finished	Maximum Time
E.2.a	Muffle Temperature		E.2.h			30 min
			E.2.l			1 hr ± 5 min
			E.2.p			10 min
			E.2.q			1 hr ± 5 min
				Standard Curve: m =		; b =

Sample ID	Sample Wt ± 0.1 g	E.1.a.ii		E.2.c		E.2.g,h		E.2.n		E.2.o		Absorbance	PPM
		Yes	No	Yes	No	Yes	No	Yes	No	Yes	No		
1. Reagent Blank													
2. Curve Check Unfortified													
3. Curve Check Fortified													
4.													
5.													
6.													
7.													
8.													
9.													
10.													

Curve Check for This Sample Set

Standard Absorbance		Within Tolerance	Yes _____	No _____
Reagent Blank Absorbance		Within Tolerance	Yes _____	No _____

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability CV %*	*Reproducibility CV %*
Arsenic (Organic/ Inorganic)	0-3.0†	90-105‡	≤10	≤20

†Based on 10 g sample.
‡Compared to standard curve prepared in sample matrix.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Absorbance of reagent blank analyzed with each set (on worksheet)	Absorbance should be ≤0.15 Abs. units. If greater, check for glassware cleanliness and contaminated reagents.
Sample weight (on worksheet)	5.0 or 10.0 g ± 0.1 g (curve must be prepared with same sample size).
Yes or no (on worksheet)	Check repipet to assure correct volume of $Mg(NO_3)_2 \cdot 6H_2O$ is added.
Final temperature of muffle (on worksheet)	<600° C
Yes or no (on worksheet)	No visible carbon.
Yes or no (on worksheet)	2.0 mL KI added.
Yes or no (on worksheet)	1.0 mL stannous chloride added.
Time started; time finished (on worksheet)	Elapsed time for standing 15-30 min.
Time started; time finished (on worksheet)	Elapsed distillation time 1 hr ± 5 min
Yes or no (on worksheet)	0.5 mL ammonium molybdate added.
Yes or no (on worksheet)	0.3 mL hydrazine sulfate added.
Time started; time finished (on worksheet)	10 min from time bath regains temperature of 95° C.
Time started; time finished (on worksheet)	60 min ± 5 min.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	% recovery	CV of recovery ≤2%; range of standard curve prepared in matrix, 80-100% (running average of last 10).
	Slope of curve	Repeatable, with ≤3% CV.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—6 levels, 3 replicates each (curves with no tissue-unashed).

0.0 μg; 5.0 μg; 10.0 μg; 15.0 μg; 20.0 μg; 25.0 μg.

ii. Phase II: Fortified samples—Use the same blank tissue throughout. It must produce ≤ 0.15 abs. units when analyzed: 5 levels, 3 replicates at the same normal levels as in Phase I, over a minimum of three separate days.

NOTE: Phase I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples from FSIS Science Western Laboratory (samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

i. Blank solution and blank tissue <0.15 abs. units.

ii. Standard curve S ≤0.05 (for each individual curve).

iii. Slopes of each curve

(a) Range 2.6000-2.8000

(b) CV: ≤3% (on last 10)

iv. See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency—initially, minimum of 1 per set, reduced to 1 per week per analyst. This sample is an internal check sample. It is a pooled sample analyzed at least 10 times to obtain a ''running'' average.

A recovery (or recoveries) is analyzed with each set of samples. Also, a ''blank'' tissue is analyzed, and a running average is maintained for both the blank and recovery. Records are maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop analysis of official samples.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above.

5. Sample Acceptablility and Stability

a. Matrices: Liver, kidney, muscle.

b. Sample receipt size: Varied; enough to obtain matrix for all required quantitative tests and reserve sample.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage:

i. Time: Indefinite.

ii. Condition: Frozen.

6. Sample Set

a. Reagent blank.

b. Tissue blank.

c. Tissue blank fortified at tolerance level.

d. Sample(s) processed on the same day for the same matrix.

7. Sensitivity

a. Lowest detectable level (LDL): 0.25 ppm.

b. Lowest reliable quantitation (LRQ): 0.05 ppm.

c. Minimum proficiency level (MPL): 0.2 ppm.

ARSENIC AND TIN

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 7
F. Analytical Quantitation 14
G. Calculations 15
H. Hazard Analysis 16
I. Worksheets 17
J. Quality Assurance Plan 23

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

This method is appropriate for the analysis of arsenic (As) and tin (Sn) in animal tissues. These elements are obtained from the tissue by ashing at an elevated temperature in a suitable furnace. The sample matrix is first ashed in the furnace and then a second heat treatment is used to remove all remaining amounts of organic residue. The ash residue is dissolved in nitric or sulfuric acid, which is then converted to its volatile hydride by sodium borohydride pellets. The hydride is purged continuously by argon into an appropriate atomizer of an atomic absorption spectrometer and converted to the gas-phase atoms.

2. Applicability

This method is suitable for the analysis of trace quantities of arsenic or tin in the liver, kidney, or muscle of all animal species.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Forced-air drying oven capable of maintaining a temperature of 95° C ± 5° C.

b. 50 mL ashing dishes, Vycor brand or equivalent (inner surfaces should not be etched).

c. Muffle furnace capable of maintaining a temperature of 550° C ± 10° C.

d. Hot plate capable of maintaining a surface temperature of 120° C ± 10° C.

e. 100 mL or 250 mL polyethylene bottles suitable for storing standard solutions.

f. Check valve replacement for nebulizer (Kontes #K-931000 or equivalent) with 6 mm capillary tubing. See Figure 1, ASN-3.

g. Erlenmeyer flasks, 125 mL standard taper 24/40.

h. Connecting adapter, 90° angle, standard taper stopcock, standard taper inner joint (Pyrex brand, Corning #9120, or equivalent). See Figure 2, ASN-3.

i. 3/8″ × 1/4″ I. D. Tygon tubing of sufficient length to extend from the Erlenmeyer flask hydride generator to the burner assembly check valve.

2. Instrument

Atomic absorption spectrophotometer equipped with background correction capability, appropriate multielement or single element hollow cathode lamp, and a three-slot argon-hydrogen entrained air burner assembly. Perkin-Elmer model 603 or equivalent.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

Figure 1

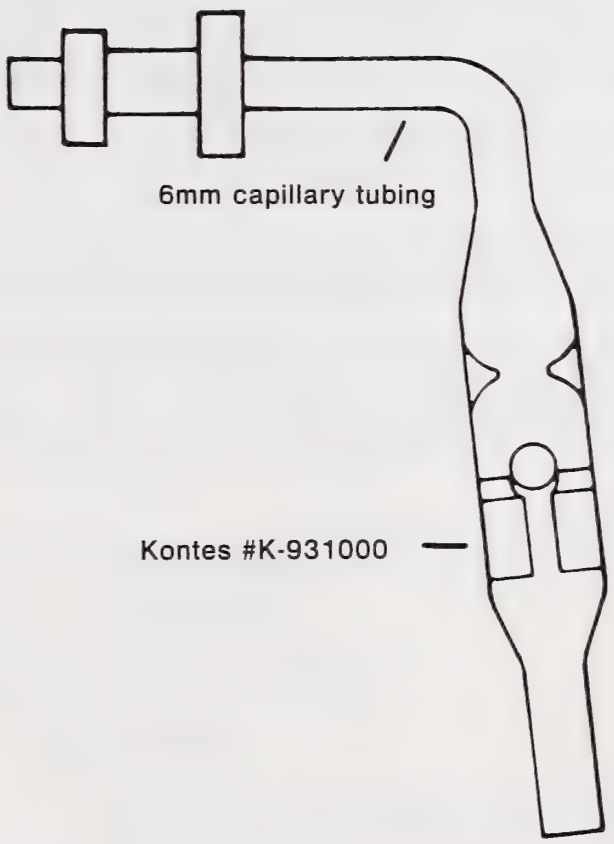
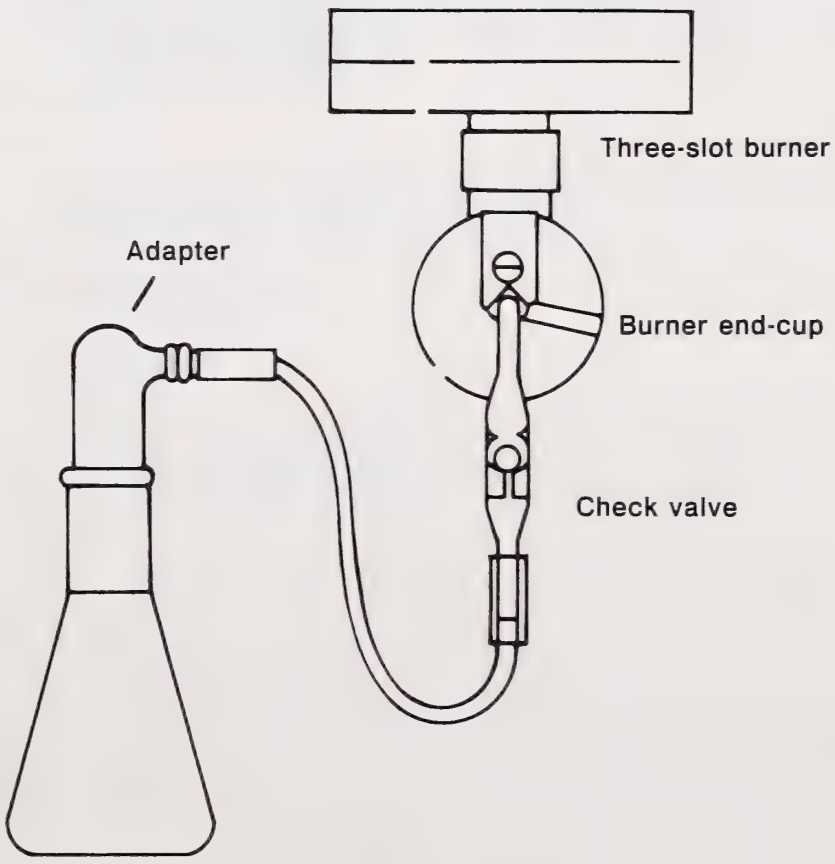


Figure 2



DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Magnesium nitrate hexahydrate solution (6.67% w/v): Dissolve 133.4 g $Mg(NO_3)_2 \cdot 6H_2O$ to 2 liters with distilled/deionized water.

b. Magnesium nitrate hexahydrate solution (50% w/v): Dissolve 500 g $Mg(NO_3)_2 \cdot 6H_2O$ to 1 liter with distilled/deionized water.

c. Nitric acid, 50%: Prepare mixture of one part concentrated nitric acid and one part distilled/deionized water.

d. Hydrochloric acid, 4.5 N: To a 1000 mL volumetric flask, add 372 mL concentrated hydrochloric acid and bring to volume at room temperature.

e. Potassium iodide solution: Prepare 15% solution (w/v) one hour before use.

f. Sodium tetrahydride borate pellets: 10/32", 98% Alfa stock #14122 (Morton Thiokol, Inc.—Alfa Products) or equivalent.

g. Sulfuric acid: Concentrated.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

All standard solutions are made from commercially available reference standard solutions. Use Fisher Certified Atomic Absorption Standards (or equivalent) for the tin and arsenic standards used in this procedure.

2. Preparation of Standards

a. Stock standards—Concentrations of commercial standard solutions are typically 1000 μg/mL (i.e., 1000 ppm).

i. Arsenic

(a) 1000 μg/mL standard inorganic arsenic solution: Fisher Cat. #SO-A-499 or equivalent.

(b) 1000 μg/mL standard organic arsenic solution as arsenilic acid: Fisher Cat. #1369 or equivalent. Dissolve 0.2897 g arsenilic acid (100% purity) in distilled/deionized water and dilute to 100 mL.

ii. Tin

1000 μg/mL standard tin solution: Alfa Products Cat. #88112 or equivalent.

b. Intermediate standards

i. Arsenic

100 μg/mL arsenic solution: Dilute 10 mL of either 1000 μg/mL arsenic solutions above to 100 mL with 4.5 N HCl.

NOTE: Because the form of arsenic used for raising animals and poultry is organic, the use of the organic arsenic standard stock solution is preferable.

ii. Tin

100 μg/mL tin solution: Add 1 mL of 1000 μg/mL standard tin solution to 10 mL volumetric flask. Dilute to volume with 10% H_2SO_4 solution.

c. Calibration standards

i. Arsenic

Dilute 1, 2, 3, 4, 5, 6, 7, 8, and 10 mL of the 100 μg/mL arsenic solution to 100 mL with 4.5 N HCl to give 1, 2, 3, 4, 5, 6, 7, 8, and 10 μg/mL standards respectively.

DETERMINATIVE METHOD

D. STANDARDS (Continued)

ii. Tin

Dilute 0.5, 1, 2, and 3 mL of the 100 μg/mL tin solution to 100 mL volumetric flasks. Dilute to volume with the 10% H_2SO_4 solution to yield 0.5, 1, 2, and 3 μg/mL standards respectively.

NOTE: Tin appears to be unstable in neutral solution and shows some decrease in stability in weakly acidic solutions. Therefore it is recommended that the stock and calibration solutions be made immediately before use.

d. Sample fortification standards

i. Arsenic

Use 1.0 mL of 4 μg/mL standard to fortify 5 g of sample tissue.

ii. Tin

Use 1.0 mL of 2 μg/mL standard to fortify 5 g of sample tissue.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Muscle

Trim off as much fat as possible. Pass muscle tissue rapidly 3 times through a food chopper with plate openings no greater than 1/8". Mix thoroughly after each chopping.

b. Liver or Kidney

Trim off as much connective tissue as possible. Place tissue into a blending jar and blend until the tissue is homogenized. Do not blend continuously for periods exceeding 1 minute. Excessive blending may overheat the tissue. Allow tissue to cool between blendings.

NOTE: Freeze samples if they are not to be analyzed immediately upon receipt. It is convenient to use flat plastic freezer bags to freeze samples in slabs 1 to 2 cm thick.

2. Ashing Procedure

a. Weigh 5.0 g ground sample into a 50 mL Vycor crucible. For arsenic samples, add 3 mL of 50% magnesium nitrate solution and mix thoroughly. For tin samples, add 5 mL of 6.67% w/v magnesium nitrate solution and mix thoroughly.

b. Spread the mixture in an even layer around the sides of the crucible. Dry in a drying oven set at 90-95° C until the sample is thoroughly dry (approximately 6 hours or overnight).

c. Place the samples into a cool (< 80° C) muffle furnace. Raise the temperature slowly (2-4° C/min) to 150° C. Maintain at this temperature until the smoking ceases. Slowly raise the temperature to 500-550° C. (See Muffle Furnace Heating Profile, ASN-8.) The sample must not be heated rapidly enough that it ignites. Hold at 500-550° C for 8-16 hours or overnight. Remove the sample from the oven and allow to cool to room temperature.

d. A second ashing step is required to remove any remaining carbon residue.

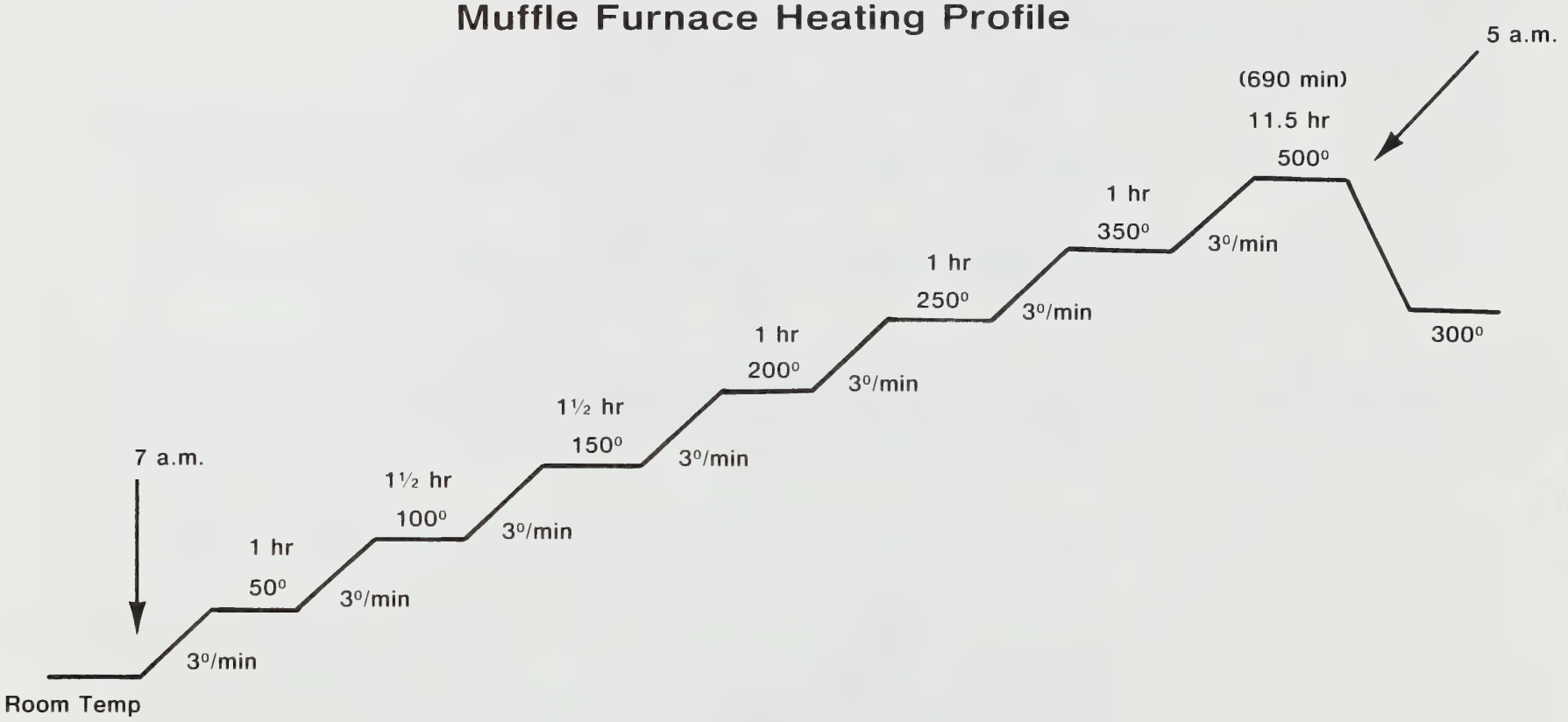
i. Arsenic: Wet the ashes with 50% nitric acid. The arsenic samples have less carbon residue due to the higher concentration of the magnesium nitrate solution used. Usually the ash only needs to be thoroughly wetted with 50% nitric acid in order to produce a carbon-free ash.

ii. Tin: Add 2 mL of 50% aq. nitric acid while washing down the sides of the crucible. Place the sample onto a hot plate set at 120° C to remove excess acid. Replace the sample in the cool muffle furnace and raise to 500-550° C. Maintain the sample at this temperature for 1 hour. Repeat this procedure until a carbon-free ash remains.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

Muffle Furnace Heating Profile



Temperatures are expressed in degrees Celsius.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Sample Transfer

a. Arsenic

i. Transfer the ash from the crucible to a clean 125 mL Erlenmeyer flask using four 10 mL-portions of 4.5 N hydrochloric acid.

ii. To this solution, add 2 mL of 15% potassium iodide solution and mix well; let stand for 20 minutes before analysis.

b. Tin

i. Moisten the ash with approximately 2 mL of 10% aq. H_2SO_4. Swirl to dissolve and transfer to a 125 mL Erlenmeyer flask. To the crucible, add 12.5 mL distilled water and 1 mL conc. H_2SO_4. Swirl to dissolve any remaining ash and transfer to the same flask. Rinse the crucible with two 12.5 mL portions of distilled/deionized water and combine them in the same flask.

NOTE: If initial dissolution is difficult, add 2-3 drops of conc. H_2SO_4 to the solution to hasten dissolution.

ii. To this solution, add 2 mL of 15% potassium iodide solution and mix well; let stand 20 minutes before analysis.

4. Preparation of Standard Curve

a. Arsenic

i. Into each of twenty 125 mL Erlenmeyer flasks, pipet 40 mL of 4.5 N hydrochloric acid.

ii. Into the flasks measure in duplicate the following: 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 10.0 μg of arsenic from the calibration standards.

iii. Add to these solutions 2 mL potassium iodide and mix well; let stand 20 minutes before analysis.

b. Tin

i. Into 125 mL Erlenmeyer flasks, measure in duplicate the following: 0, 0.5, 1.0, 2.0, and 3.0 μg of tin from the calibration standards.

ii. To each flask, add 50 mL distilled/deionized water and mix.

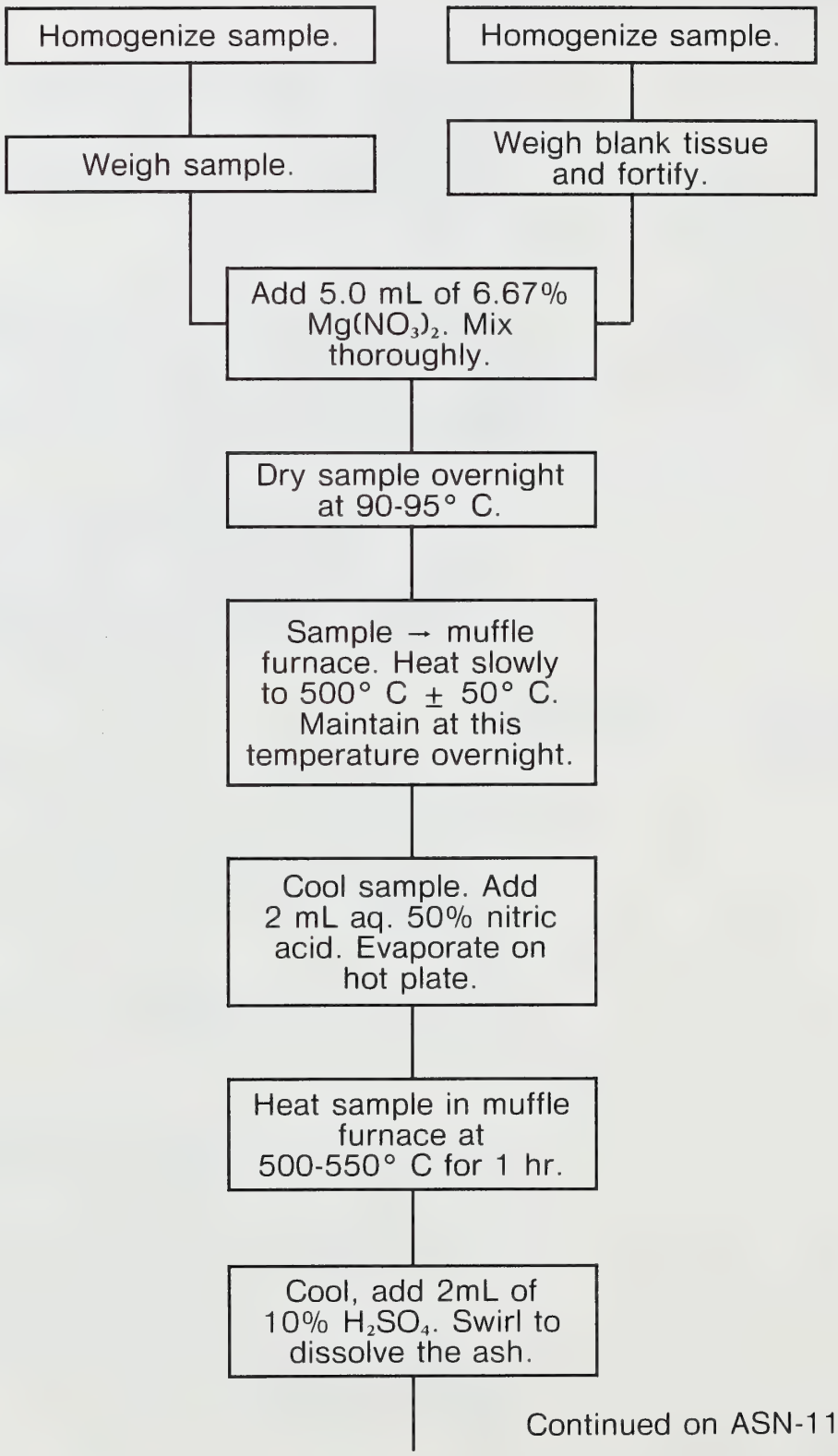
iii. Add 1.0 mL concentrated sulfuric acid to each flask and mix well.

iv. Add 2mL of 15% potassium iodide to each flask; let stand 20 minutes before analysis.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

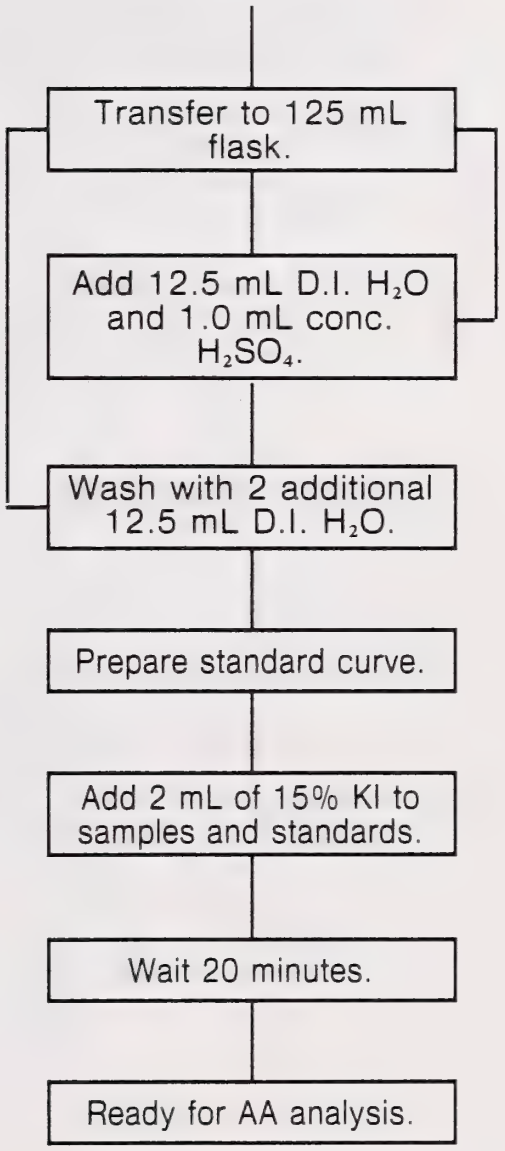
5. Flow Chart Summary
Tin Analysis
[Muscle, Liver, Kidney]



Continued on ASN-11

DETERMINATIVE METHOD

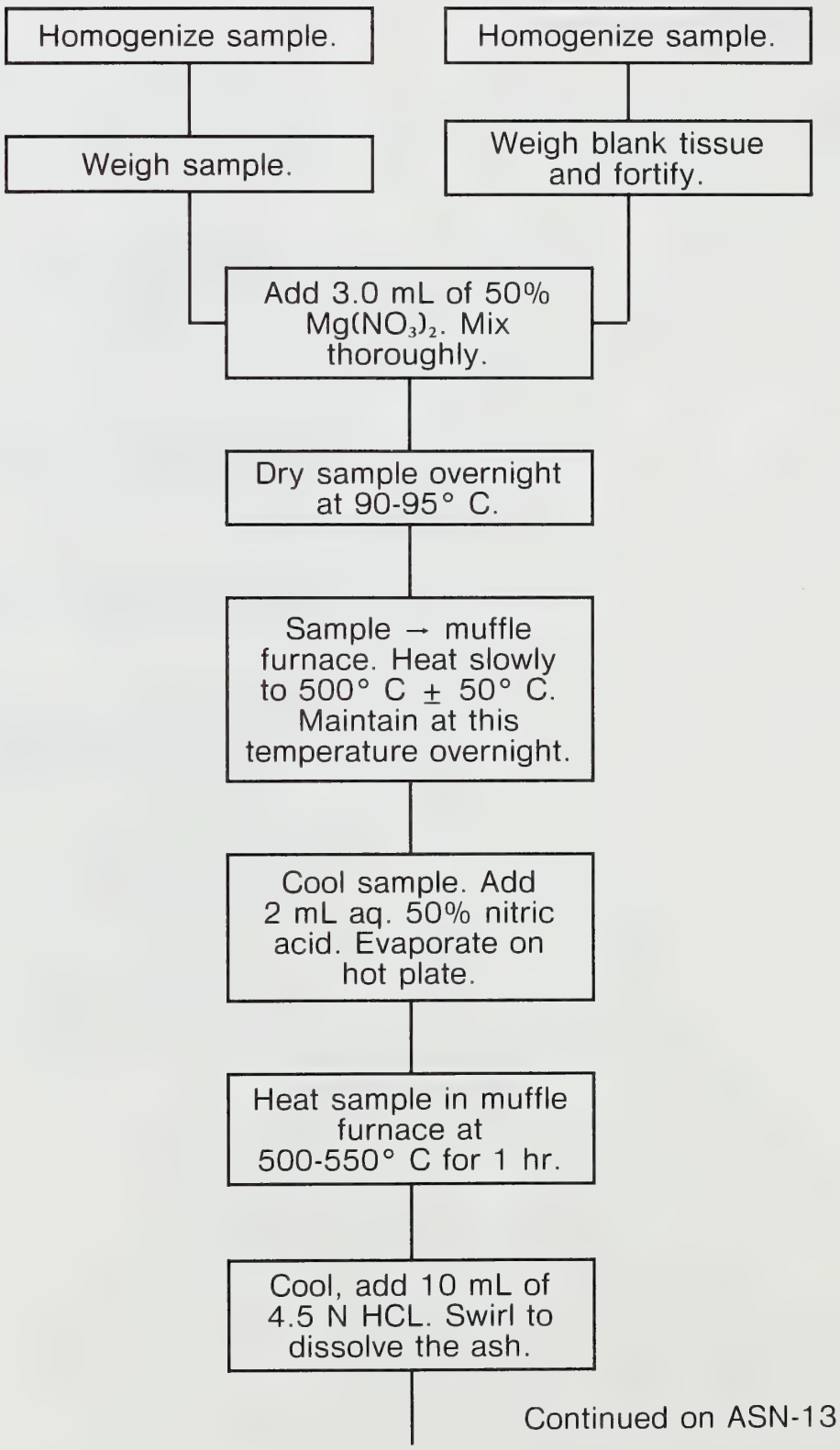
E. EXTRACTION PROCEDURE (Continued)



DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

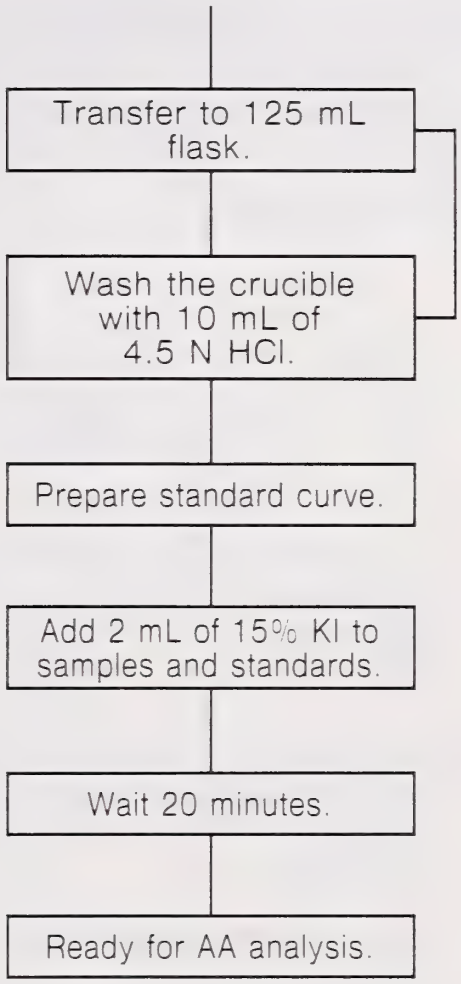
5. Flow Chart Summary
Arsenic Analysis
[Muscle, Liver, Kidney]



Continued on ASN-13

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

Set up the AA spectrophotometer according to the manufacturer's instructions. Insert an arsenic or tin hollow cathode lamp and maximize the signal intensity by adjusting the position of the lamp. Make a fine tuning of the wavelength by maximizing the signal. Adjust the height of the burner. Ignite the hydrogen-argon-entrained air flame and aspirate an arsenic or tin standard (e.g., 5 μg/mL) into the flame. Adjust the aspiration rate and position of the burner head for maximum signal.

Gas Flow Conditions

Gas	*Tank Pressure*	*Regulated Pressure*	*Flow*
Ar	50 lbs	30 lbs	60 ml/min
H_2	15 lbs	8 lbs	24 ml/min

Monochrometer Setting

Element	*Wavelength (nm)*	*Slit Width*	*Expansion*
As	193.7	4	0.35
Sn	224.3	4	0.65

2. General Operation

a. Begin analyses with the highest point on one of the standard curves. Add sodium borohydride pellets by placing them in the adapter (Figure 2, ASN-3) and letting them drop into the sample as the connection is made.

Note: Arsenic, 2 pellets; tin, 3 pellets.

b. Measure peak height and plot standard curves as appropriate.

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Standard curves are either calculated by least mean squares, for the linear range only, or are plotted from the standard data obtained. The arsenic or tin of each sample is then determined from the standard curves.

Arsenic: The calibration curve should be linear up to 4.0 μg, with some curvature at higher arsenic levels.

Tin: The calibration curve should be linear up to 3.0 μg of tin, with some curvature at higher tin levels.

Sample calculation:

$$\text{actual ppm} = \frac{\mu\text{g from standard curve}}{\text{sample weight (g)}}$$

$$\text{corrected ppm} = \frac{\text{actual ppm}}{\text{recovery of fortified tissue}}$$

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Arsenic and Tin Analysis by Atomic Absorption Spectrometry		
2. Required Protective Equipment	Safety glasses, plastic gloves, laboratory jacket or coat, heat-resistant gloves, crucible tongs.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	B. Equipment		
	Muffle furnace	Hot!	Wear heat-resistant gloves. Use crucible tongs to remove and insert crucible.
	C. Reagents		
	Nitric acid Hydrochloric acid Sulfuric acid	Corrosive. Contact with fumes or liquid can result in burns and severe skin, eye, and respiratory irritation.	All steps using acid should be performed in a hood. Wear plastic gloves.
	Sodium tetrahydroborate	Flammable solid. Releases hydrogen on contact with acid or water. Causes burns on contact with skin, eyes, or respiratory areas.	Avoid accidental contact with moisture. Wear plastic gloves while handling pellets. Wash affected areas with plenty of water.
4. Disposal Procedures	Sample, standard solution, and reagents.	See above	All solutions should be flushed down the drain with lots of water.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheets on the following pages, *Arsenic Analysis by Atomic Absorption* and *Tin Analysis by Atomic Absorption*, can be removed from this book for photocopying whenever necessary, but do not forget to replace them.

ARSENIC ANALYSIS BY ATOMIC ABSORPTION

ANALYST _____

DATE STARTED _____

DATE COMPLETED _____

INSTRUMENT CONDITIONS

Wavelength (nm)—193.7
Slit Width—4
Expansion—0.35
Flow Rates—Argon 60 cc/min
Hydrogen 24 cc/min

STANDARDS AND CURVE DATA

Total μg As	Peak Hght #1	Peak Hght #2	Avg Peak Hght
0.0			
1.0			
2.0			
3.0			
4.0			
5.0			
6.0			
7.0			
8.0			
10.0			

	YES	NO
Section E.3.a.i. 10 mL	☐	☐
Section E.3.a.ii. and Section E.4.a.iii. 2mL	☐	☐
Section E.4.a.i. 40 mL	☐	☐

Sample Identification	Tissue Code	Sample Weight	Peak Height	Total μg As	ppm
Control					
Fortified					

Sample Identification	Tissue Code	Sample Weight	Peak Height	Total μg As	ppm

TIN ANALYSIS BY ATOMIC ABSORPTION

ANALYST _____

DATE STARTED _____

DATE COMPLETED _____

INSTRUMENT CONDITIONS

Wavelength (nm)—224.3
Slit Width—4
Expansion—0.65
Flow Rates—Argon 60 cc/min
Hydrogen 24 cc/min

STANDARDS AND CURVE DATA

Total μg Sn	Peak Hght #1	Peak Hght #2	Avg Peak Hght
0.0			
0.5			
1.0			
2.0			
3.0			

LINEAR REGRESSION LINE

Slope, m =
Intercept, b =
Correlation Coef., r =
STD, Error of Estimate, s =

	YES	NO
Section E.3.b.i. 12.5 mL	☐	☐
Section E.3.b.i. and Section E.4.b.iii. 1.0 mL	☐	☐
Section E.4.b.iv. 2 mL	☐	☐

Sample Identification	Tissue Code	Sample Weight	Peak Height	Total μg Sn	ppm
Control					
Fortified					

Sample Identification	Tissue Code	Sample Weight	Peak Height	Total μg Sn	ppm

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Element	*Analytical Range (ppm)**	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
As (org.)	0-2	70-110	≤ 10	≤ 20
As (inorg.)	0-2	80-110	≤ 10	≤ 20
Sn (inorg.)	0-0.6	80-110	≤ 10	≤ 20

* ppm = based on 5.0 g sample.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5.0 ± 0.1 g
b. Start with a cool muffle furnace	Yes
c. Raise furnace temperature slowly	Yes
d. Final temperature	500° C ± 50° C
e. No visible carbon	Yes
f. Reagent blank	Absorbance should be < 0.15 Abs. units or show response < 0.3 μg. If greater, check for cleanliness and contaminated glassware.
g. Standards	New commercial standards should be verified against old standards (agree within ± 10%).

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards—3 replicates each of the following standards on 3 consecutive days (curves with no tissue—unashed).

Arsenic		*Tin*	
(μg)	*(ppm)**	*(μg)*	*(ppm)**
0.0	0.0	0.0	0.0
1.0	0.2	0.5	0.1
2.0	0.4	1.0	0.2
3.0	0.6	2.0	0.4
4.0	0.8	3.0	0.6
5.0	1.0		
6.0	1.2		
7.0	1.4		
8.0	1.6		
10.0	2.0		

*ppm = calculated based on 5.0 g sample weight

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

ii. Phase II: Fortified samples—Use the same blank tissue throughout. It must produce ≤ 0.15 absorbance units when analyzed; duplicate analysis on four or five levels of Phase I on 3 separate days, one set each for each type of matrix.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Fourteen samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability Criteria

Refer to Section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Initially a minimum of 1 check sample per set, reduced to once per week per analyst. This sample is an internal check sample. It is a pooled sample analyzed at least 10 times to obtain a running average.

ii. A recovery (or recoveries) is analyzed with each set of samples. Also, a blank tissue sample is analyzed and a running average is maintained for both the blank and the recovery (on the last 10). Records are maintained by the analysts and are reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings

(b) Control chart on differences between replicates

(c) All percent recoveries

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability

a. Matrices: Liver, kidney, muscle.

b. Sample receipt size: Approximately 25 g.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage

i. Time: 6 months

ii. Condition: Frozen (< −10° C)

6. Sample Set

Each set must include a reagent blank, a blank tissue, a blank tissue fortified at the concentration level of interest, and up to 20 samples.

NOTE: Reagent blank is required to help determine if there is any trace of contamination from glassware or reagent used.

7. Sensitivity

a. Lowest detectable level (LDL):

Arsenic		*Tin*	
μg	*ppm*	*μg*	*ppm*
0.5	0.1	0.25	0.05

b. Lowest reliable quantitation (LRQ):

Arsenic		*Tin*	
μg	*ppm*	*μg*	*ppm*
1.0	0.2	0.5	0.1

c. Minimum proficiency level (MPL): Not applicable.

BENZIMIDAZOLES

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 5
C. Reagents and Solutions 7
D. Standards 8
E. Extraction Procedure 11
F. Analytical Quantitation 16
G. Calculations 23
H. Hazard Analysis 24
I. Worksheet 25
J. Quality Assurance Plan 29

II. Confirmatory Method

A. Introduction 32
B. Equipment 32
C. Reagents 32
D. Standards 33
E. Sample Preparation and Cleanup 33
F. Analytical Confirmation Procedure 35
G. [Reserved]
H. Hazard Analysis 55
I. Worksheet 55
J. Quality Assurance Plan 59

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structures

This procedure is for the determination of benzimidazole drug residues using reverse-phase high performance liquid chromatography (HPLC) with ultraviolet absorbance detection. Residues that can be determined by this procedure include thiabendazole (TBZ) and its metabolite 5-hydroxythiabendazole (5-OH TBZ), albendazole 2-aminosulfone metabolite (ALB), benomyl in the active hydrolyzed form carbendazim (BEN), oxfendazole (OFZ), mebendazole (MBZ), cambendazole (CAM), and fenbendazole (FBZ). The sensitivity of the method is approximately 25 ppb based on a 10-gram initial sample. The method exploits the weakly basic nature of the target benzimidazoles for effective extraction and cleanup. A 10-gram sample of ground tissue is fortified with 2-(n-butylmercapto) benzimidazole as the internal standard and shaken with granular sodium sulfate, 4M potassium carbonate, and ethyl acetate. The mixture is centrifuged and the liquid transferred to another tube. The volume is reduced and transferred quantitatively with ethyl acetate to a smaller tube and evaporated to dryness. The dried residue is dissolved in hexane. The hexane solution is partitioned into an ethanol/0.2N HCl solution and centrifuged. The hexane layer is removed and discarded. An aliquot of the aqueous phase is dried, and the dried residue is dissolved in mobile phase for HPLC analysis. The target benzimidazoles are separated and quantified by HPLC using UV absorbance at 298 nanometers for detection. Poultry tissues are examined at 280 nanometers for carbendazim.

For samples where matrix effects prevent satisfactory HPLC analysis, an aliquot of the ethanol/0.2N HCl solution is applied to a reverse C2 solid phase extraction (SPE) column. The benzimidazoles are eluted with ethyl acetate. The eluate is evaporated and the residue dissolved in mobile phase for injection onto the HPLC column.

Thiabendazole: $C_{10}H_7N_3S$
F.W. = 201.3

5-Hydroxythiabendazole: $C_{10}H_7N_3OS$
F.W. = 217.3

2-Aminosulfone albendazole metabolite: $C_{12}H_{15}N_3O_3S$
F.W. = 239.3

Oxfendazole: $C_{15}H_{13}N_3O_3S$
F.W. = 315.4

Mebendazole: $C_{16}H_{13}N_3O_3$
F.W. = 295.3

Cambendazole: $C_{14}H_{14}N_4O_2S$
F.W. = 302.4

Fenbendazole: $C_{15}H_{13}N_3O_2S$
F.W. = 299.4

Carbendazim: $C_9H_9N_3O_2$
F.W. = 191.2

DETERMINATIVE METHOD

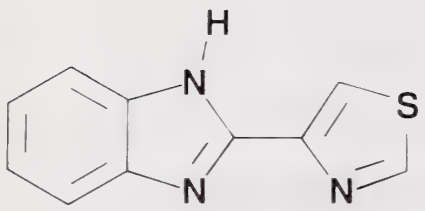
A. INTRODUCTION (Continued)

2. Applicability

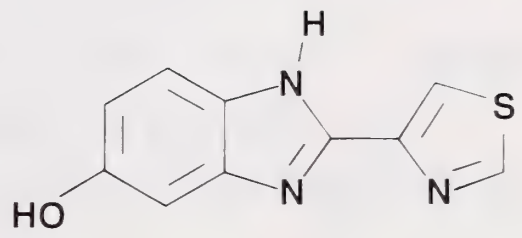
Benzimidazole anthelmintics are widely used in animal production to control internal worm parasites. This method is for benzamidizole analysis in swine, bovine, and ovine liver and muscle, and in processed pork products. The procedure can also be used to analyze poultry tissues for carbendazim (BEN).

DETERMINATIVE METHOD

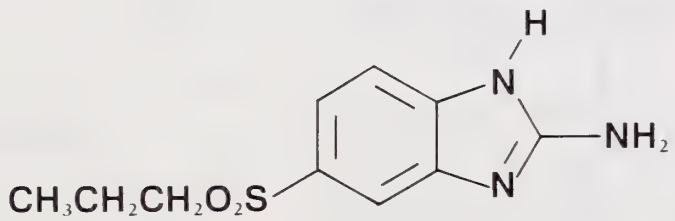
A. INTRODUCTION (Continued)



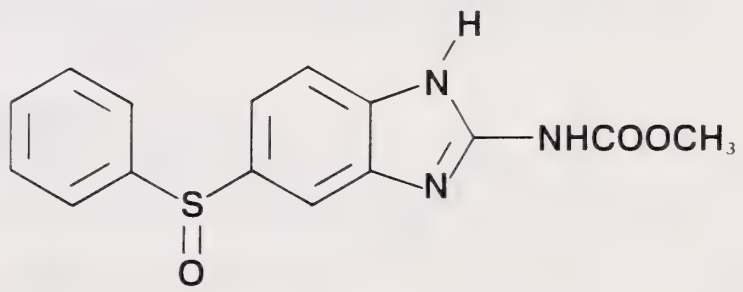
THIABENDAZOLE



5-HYDROXYTHIABENDAZOLE (THIABENDAZOLE METABOLITE)



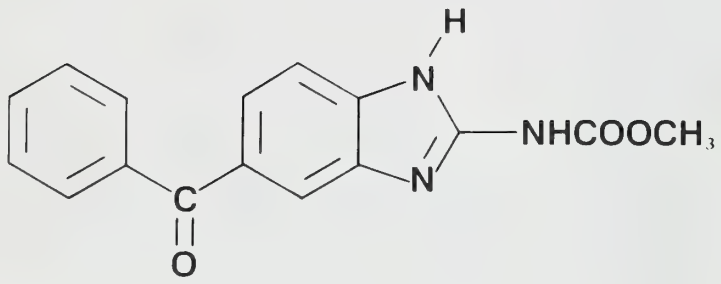
2-AMINOSULFONE ALBENDAZOLE METABOLITE



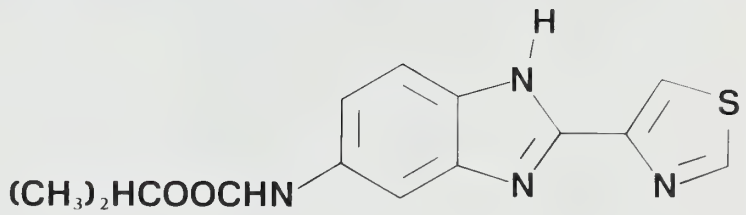
OXFENDAZOLE

I. DETERMINATIVE METHOD

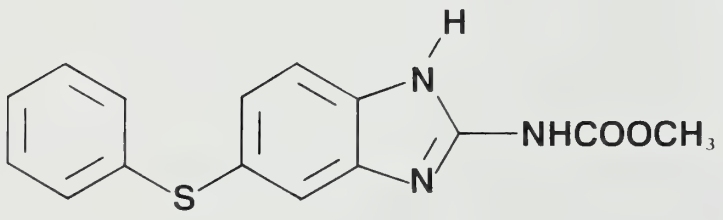
A. INTRODUCTION (Continued)



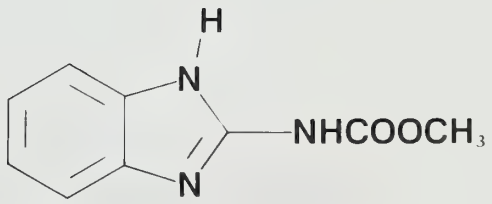
MEBENDAZOLE



CAMBENDAZOLE



FENBENDAZOLE



CARBENDAZIM

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Analytical columns. All columns listed have been used successfully.

   i. 12.5 cm × 4.6 mm i.d., Whatman PartiSphere Cartridge, C18, 5 μm.

   ii. 15 cm × 4.6 mm i.d., Econosphere, C18, 3 μM, Alltech.

   iii. 25 cm × 4.6 mm i.d., Hibar Lichosorb, C18, 5 μM, EM Science.

b. Platform mechanical shaker, Eberbach Corp., or equivalent.

c. Centrifuge tubes, 50 mL plastic, disposable, Falcon (Becton Dickinson), or equivalent.

d. Centrifuge, Damon/IEC Division Model HN-SII, or equivalent.

e. IEC Model #58 centrifuge rotor, with Model #325 15 mL and Model #310 50 mL cups.

f. N-Evap, Organomation Associates, or equivalent.

g. Centrifuge tubes, 15 mL glass, disposable (Kimble #73785) and screw caps with PTFE faced rubber liners (Kimble #73802).

h. Volumetric flasks, Class A accuracy, 10, 100, 200, and 1000 mL.

i. Glass tubes, screw-top with Teflon-lined caps, 25 × 150 mm, 50 mL capacity (Corning 9826).

j. Culture tubes, 50 mL disposable glass (Corning 99445).

k. Reacti-therm heating module, Pierce Model #18800 with evaporating unit, Pierce Model #1877780, or equivalent.

l. pH meter, Orion 701A, or equivalent.

m. Vacuum manifold device for aid in washing and elution of C2 columns. Optional but allows for multiple C2 elutions. Analytichem or equivalent.

n. Vortex mixer, Labline Model #1290, or equivalent.

o. Pasteur pipettes, 5 3/4", American Scientific Products #P5202-1 or equivalent.

p. Test tube racks.

q. Pipettes, disposable, 10 mL glass serological, American Scientific Products #4650-110.

r. Brinkman Dispensettes, adjustable, of various capacities: 1.0-5.0 mL, 2.0-10.0 mL, and 10-50 mL.

s. Volumetric pipettes, Class A accuracy, 0.5, 1, 2, 5, and 40 mL.

t. Syringe, tuberculin, 1 mL, Becton Dickinson.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

u. Syringe, gastight, 1000 μL, Hamilton #1001.

v. Balance, top-loader, Mettler PE 3600 or equivalent.

w. Balance, analytical, Mettler AK 160 or equivalent.

x. Vials, autosampler, 4 mL, compatible with Waters WISP, and limited volume inserts (300 μL), springs, open top caps, and septa.

y. Disposable filter assembly, 0.2 micron, ACRO LC 13, Gelman #4450.

2. Instrumentation

Liquid chromatograph, Waters Chromatography Division, Millipore, or equivalent, consisting of Model 6000A solvent delivery system connected to Model 712 WISP automatic sample injection module with Model 481 Lambda-Max ultraviolet absorbance detector and Model 730 data module.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Ethyl acetate, pesticide grade.

b. Ethyl alcohol, ACS reagent grade.

c. Methanol, HPLC grade.

d. Hexane-UV, HPLC grade.

e. Acetone, ACS reagent grade.

f. Dimethylsulfoxide, silylation grade.

g. Distilled water, HPLC grade.

h. Sodium sulfate, ACS reagent grade, anhydrous, granular.

i. Potassium carbonate, ACS reagent grade, anhydrous, granular. Prepare 4 M solution by dissolving 552.84 g K_2CO_3 in 1 liter distilled water.

j. Hydrochloric acid solution. Prepare 0.2N solution by diluting 16.5 mL ACS reagent grade HCl to 1 liter with distilled water.

k. Ethanol:0.2N HCl solution. Combine 66 mL alcohol (2.) with 33 mL of 0.2N HCl solution (12.). Prepare fresh solution every two weeks.

l. Potassium bicarbonate, ACS reagent grade. Prepare 2% aqueous solution with distilled water.

m. Triethylamine, 99+ percent pure (Aldrich Chemical Co.).

n. Ammonium hydroxide, ACS reagent grade. Prepare 50% aqueous solution of NH_4OH with distilled water.

o. Ammonium phosphate monobasic, ACS reagent grade. Prepare 0.01 M, pH 7.0 buffer by dissolving 1.15 g $NH_4H_2PO_4$ in approximately 950 mL distilled water, adjusting to pH 7.0 with 50% NH_4OH solution and bringing to final volume of 1 L. Prepare just before use and refrigerate.

p. Methanol:ammonium phosphate monobasic buffer mixture, without triethylamine, to be used in preparation of working standards and for reconstituting dried residue. Combine 530 mL methanol (3.), 470 mL 0.01 M $NH_4H_2PO_4$ buffer (15.). Pass through 0.45 μM filter before use.

q. HPLC mobile phase, 53:47 methanol:$NH_4H_2PO_4$ buffer containing 10 mM triethylamine. Combine 530 mL methanol (3.), 470 mL 0.01 M $NH_4H_2PO_4$ buffer (15.) and 1400 mL triethylamine (13.). Pass through 0.45 μM filter before use.

r. Prep-Elute SPE C2 (ethyl) columns, 200 mg, 3 mL capacity, (P. J. Cobert #944088).

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Drug Standards

i. Cambendazole, benomyl, thiabendazole, 5-hydroxythiabendazole —Merck and Co., Inc., 126 E. Lincoln Ave., Rahway, NJ 07065.

ii. Carbendazim (active form of benomyl)—U. S. Environmental Protection Agency, Pesticides and Industrial Chemicals Repository (MD-8), Research Triangle Park, NC 27711.

iii. Albendazole 2-aminosulfone metabolite—Smith, Kline Animal Health Products, 1600 Paoli Ave., West Chester, PA 19380. Product #81038.

iv. Oxfendazole—Syntex Opthalmics, 10210 N. 25th Ave., Phoenix, AZ 85021.

v. Mebendazole—Janssen Pharmaceuticals, 40 Kingsbridge Rd., Piscataway, NJ 08854.

vi. Fenbendazole—Hoechst-Roussel Pharmaceuticals Inc., Route 202-206 North, Somerville, NJ 07110.

b. Internal Standard

Butyl iodide (1-Iodobutane) 99% purity, and 2-Mercaptobenzimidazole 98% purity, are obtained from Aldrich. they are used to synthesize the internal standard, 2-(n-butylmercapto) benzimidazole as explained below. The internal standard is used as a within-set quality reference.

2. Preparation of Standards

a. Drug Standards

i. Prepare individual 1 mg/mL stock solutions of 2-aminosulfone albendazole, carbendazim, oxfendazole, mebendazole, and fenbendazole, using 10 mg drug standard in 10mL dimethyl sulfoxide.

ii. Prepare individual 1 mg/mL stock solutions of thiabendazole, 5-hydroxythiabendazole, and cambendazole, using 10 mg drug standard in 10 mL methanol.

iii. Prepare a mixed standard intermediate solution containing all eight drugs by combining a 2 mL aliquot of the fenbendazole stock solution with 1 mL aliquots of the stock solutions of each of the remaining drugs, in a 100 mL volumetric flask. Dilute to volume with methanol. The concentration of each drug in this mixed intermediate solution is 10 μg/mL, except for fenbendazole, which is 20 μg/mL.

DETERMINATIVE METHOD

D. STANDARDS (Continued)

iv. Prepare mixed standard working solutions by transferring 0.5, 1.0, and 2.0 aliquots of the intermediate solution into separate 10 mL volumetric flasks. Add matching volumes of 0.01 M $NH_4H_2PO_4$ buffer to the respective flasks. Dilute each flask to volume with the methanol:$NH_4H_2PO_4$ buffer solution. The concentrations of the resulting solutions will be 0.5, 1.0, and 2.0 μg/mL, except for fenbendazole (1.0, 2.0, and 4.0 μg/mL). One milliliter of 1.0 μg/mL solution in 10 g of tissue is equivalent to 100 ppb (200 ppb for fenbendazole).

b. Internal Standard

i. Preparation of stock solution of internal standard 2-(n-butyl-mercapto) benzimidazole: 200 μg/mL. Weigh 2.0 mg of 2-(n-butylmercapto) benzimidazole and place in a 50 mL screw-cap glass tube with Teflon-lined cap.

ii. Add 10 mL acetone, 200 μL n-Butyl iodide, and 100 mg potassium carbonate to the tube.

iii. Mix the contents well by vortexing, seal the tube, and reflux at 90° C for 90 minutes on a heating module.

iv. Cool the solution under cold water to room temperature.

v. Transfer the solution to a 50 mL test tube.

vi. Rinse reaction tube with 5 mL acetone and add to the test tube.

vii. Evaporate the contents of the test tube to dryness at 40° C under a gentle stream of nitrogen.

viii. Add 3 mL methanol to the tube and mix to reconstitute the residue.

ix. Transfer the solution to a 10 mL volumetric flask.

x. Wash the test tube twice with 2 mL of methanol and add both washes to the volumetric flask. Dilute the solution to the 10 mL mark with methanol.

xi. Preparation of intermediate solution of the internal standard: 10 μg/mL. Transfer a 5 mL aliquot of the stock solution of 2-(n-butylmercapto) benzimidazole to a 100 mL volumetric flask.

xii. Dilute to mark with methanol.

xiii. Preparation of working standard of the internal standard: 2 μg/mL. Transfer a 40 mL aliquot of the intermediate solution of 2-(n-butylmercapto) benzimidazole to a 200 mL volumetric flask.

I. DETERMINATIVE METHOD

D. STANDARDS (Continued)

	xiv. Dilute to mark with methanol:$NH_4H_2PO_4$ solution (53:47, no triethylamine). xv. Compare each newly synthesized standard with the previous run to assure completeness of synthesis reaction.
3. Storage Conditions	– 20° C, away from light.
4. Shelf Life Stability	Unknown at this time.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Procedure

a. Weigh 10 g of blended sample into a 50 mL disposable plastic centrifuge tube. Weigh frozen, let thaw before fortifying.

b. To each sample, add 1 mL of 2-(n-butylmercapto) benzimidazole internal standard (2.0 μg/mL in methanol:$NH_4H_2PO_4$ buffer, 200 ppb).

c. SCREENING: Prepare a fortified blank sample for each sample set. Add the internal standard to the blank tissue at 200 ppb. Fortify the recovery sample at 100 ppb for all analytes except fenbendazole (FBZ), which is fortified at 200 ppb.

REANALYSIS OF POSITIVES: Prepare a fortified blank sample for each sample set. Add the internal standard to the blank tissues at 200 ppb. Fortify three recovery samples at three different concentrations (50, 100, and 200 ppb), except fenbendazole (FBZ), which is fortified at 100, 200, and 400 ppb. Data will be used to construct a standard curve.

d. Add 5 g sodium sulfate, 1 mL potassium carbonate solution, and 30 mL ethyl acetate *in that order* to all fortified and sample tubes.

e. Cap tube and shake vigorously by hand 10 seconds; then shake 10 minutes on the mechanical shaker at high speed.

f. Centrifuge the tubes for 5 minutes at 2500 rpm (1200 g).

g. Decant the supernatant into another identically labeled 50 mL disposable plastic centrifuge tube. Do not allow any sediment to be transferred into the next tube. Remove any sediment from the new tube with a Pasteur pipette.

h. Reduce the volume to about 10 mL on an N-Evap under a gentle stream of nitrogen using a water bath temperature of approximately 55° C.

i. Transfer the remaining liquid from the 50 mL tube into a 15 mL disposable glass centrifuge tube.

j. Rinse the 50 mL tube twice with 2 mL ethyl acetate and add both rinses to the 15 mL tube.

k. Evaporate the contents of the 15 mL tube to dryness on an N-Evap at 55° C under a gentle stream of nitrogen.

l. Add 5 mL of hexane-UV grade to the dried residue and mix well for 20 seconds on the vortex mixer. (The procedure may be interrupted at this point.)

m. Add 1 mL of the ethanol:0.2N HCl solution and vortex thoroughly.

NOTE CRITICAL CONTROL POINT: The ethanol:acid solution must be made fresh every two weeks. Solutions older than two weeks will tend to convert fenbendazole to oxfendazole during analysis.

n. Centrifuge the tubes for 2 minutes at 1000 rpm (500 G) to separate the phases.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

o. Using a Pasteur pipette attached to a vacuum source via a trap, aspirate off and discard the hexane (upper phase). Avoid disturbing the ethanol:acid phase or any emulsion present at the interface.

p. Again add 5 mL of hexane-UV to the tube and vortex.

q. Centrifuge the tubes for 2 minutes at 1000 rpm (500 G) to separate the phases.

r. Aspirate and discard the hexane (upper phase) and any emulsion present.

s. Optional: If a precipitate is present, centrifuge 2 minutes at 2500 rpm (1200 G).

t. Transfer 300 μL of the ethanol:HCl solution from the tube to a 4 mL vial.

u. Evaporate this volume to dryness on a heating module at 35-40° C (low setting) under a gentle stream of nitrogen.

v. Add 300 μL of the methanol:ammonium phosphate buffer solution (53:47, without triethylamine) to the vial and mix on a vortex mixer to reconstitute the residue.

w. Transfer the volume (using 1 mL tuberculin syringe fitted with acrodisc filter) to an assembled autosampler vial with limited volume insert for HPLC injection.

NOTE: Perform the following steps if the initial chromatogram of a sample is unacceptable because of poor separation between the peaks contributed by the tissue matrix and the first two eluting peaks of interest, 2-aminosulfone albendazole (ALB) and 5-hydroxythiabendazole. Always perform these steps prior to extract being presented for GC/MS confirmation.

x. Prepare one C2 column for each sample to be re-analyzed by washing each C2 column sequentially with volumes (6 mL) of ethyl acetate, one column volume (3 mL) of ethanol and one column volume (3 mL) of distilled water. Pass each wash through using low vacuum and discard the washes. Do not allow the column packing to dry between washes. Plug (with cap from 1 mL tuberculin syringe) the outlet of the C2 column at the end of the water wash, leaving a drop of water above the column packing. The conditioned C2 column is set aside until used in step aa.

y. Transfer 300 μL of the ethanol:acid solution (from step r) to a 15 mL disposable glass centrifuge tube.

z. Add 2 mL of the 2% aqueous potassium bicarbonate solution to the tube and mix thoroughly (up to 2 minutes) on the vortex mixer.

aa. Apply the entire volume from the tube to the conditioned C2 volume and allow the volume to pass through by gravity.

bb. Wash the C2 column twice with distilled water and then dry the column packing using low vacuum until visible interstitial water is absent.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

cc. Elute the adsorbed analytes from the C2 column by passing 900 μL ethyl acetate through by gravity. The final volume may be eluted from the column by applying positive pressure with a 20 mL plastic syringe or a pipette bulb. Collect the eluate in a 4 mL vial.

dd. Evaporate the eluate to dryness on a heating module at 35-40° C (low setting) under a gentle stream of nitrogen.

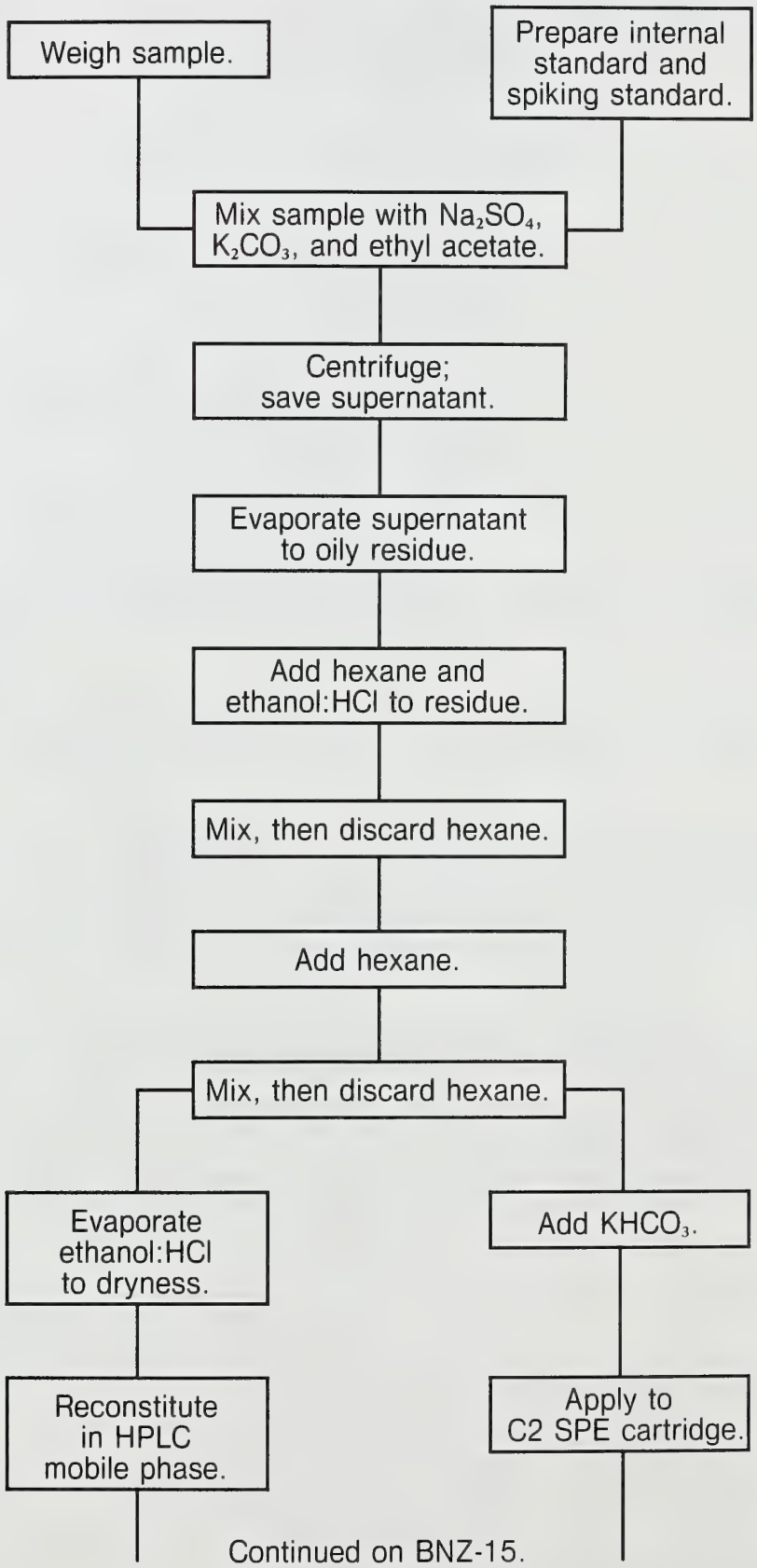
ee. Add 300 μL of the methanol:ammonium phosphate buffer solution (53:47, no triethylamine) to the vial and mix on a vortex mixer to reconstitute the residue.

ff. Transfer the volume, using a 1 mL tuberculin syringe fitted with an acrodisc filter, to an assembled autosampler vial with limited volume (300 μL) insert for HPLC injection.

I. DETERMINATIVE METHOD

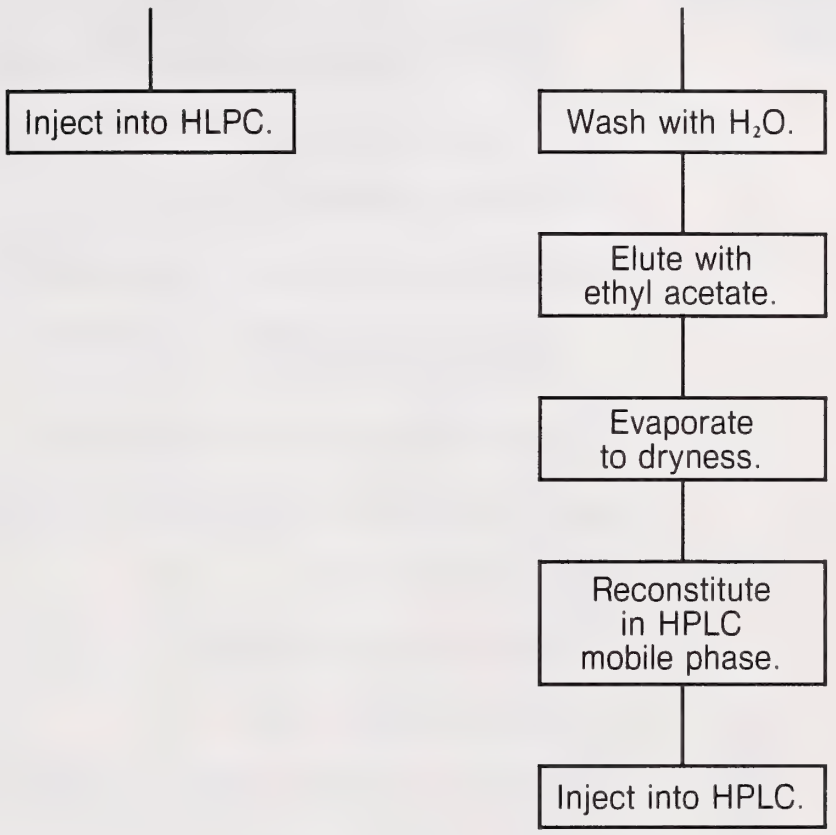
E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary



I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. Mobile phase: Methanol:$NH_4H_2PO_4$ buffer (53:47) with 10 millimolar triethylamine, run isocratically. Use 40:60 methanol:$NH_4H_2PO_4$ buffer with 10 millimolar triethylamine for carbendazim in poultry tissue.

b. Flow rate: 0.75 mL/min (1400-2500 psi)

c. Injection volume: 20 μL

d. Column temperature: ambient (22° C)

e. Detector absorbance wavelength: 298 nm; 280 nm for carbendazim in poultry tissues

f. Detector sensitivity: 0.02 AUFS

g. Data module chart speed: 1.00 cm/min

h. Peak width: 24

i. Noise rejection: 6.800

j. Area rejection: 100

k. Calculations: manually, by peak height

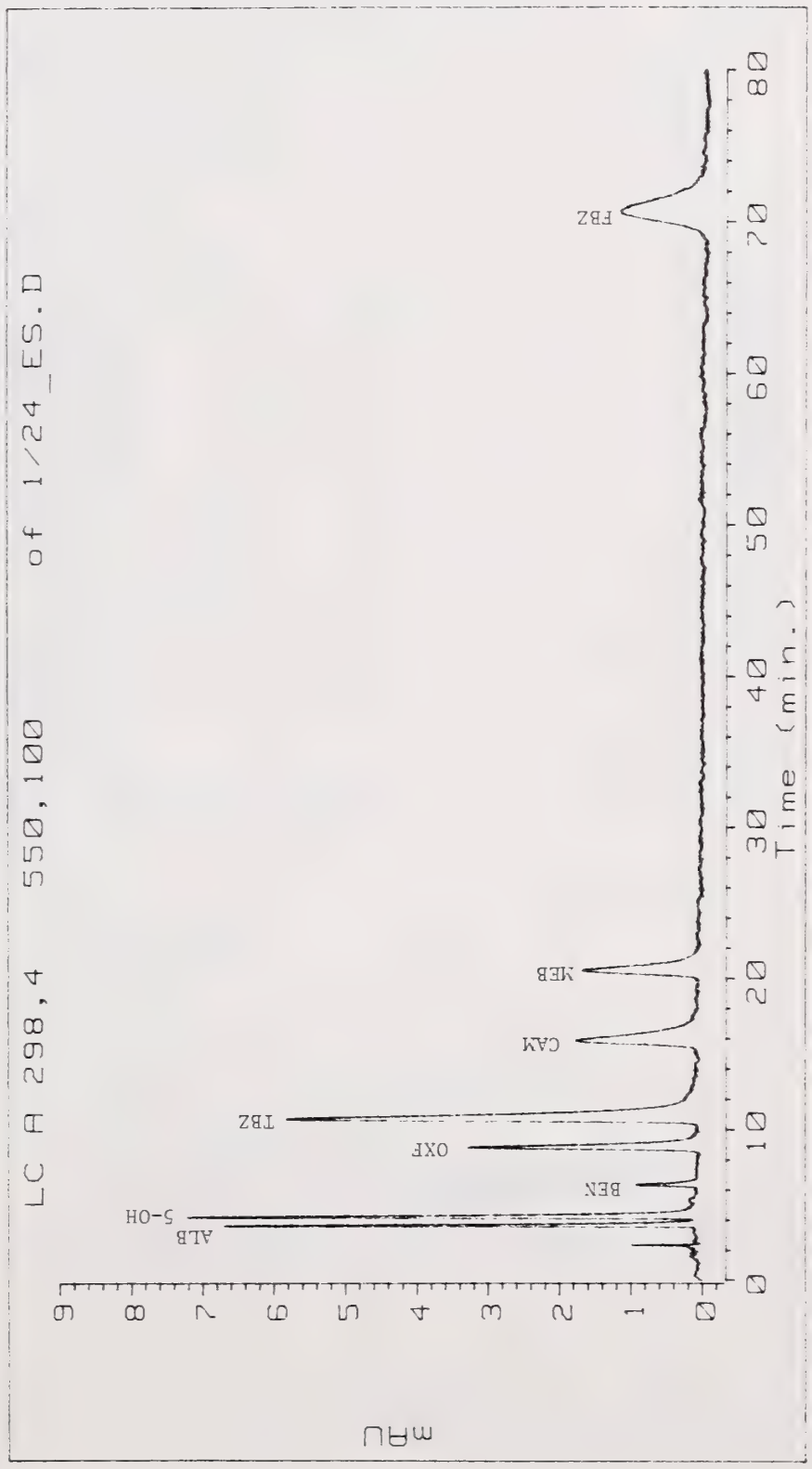
2. Interferences

None known at this time.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

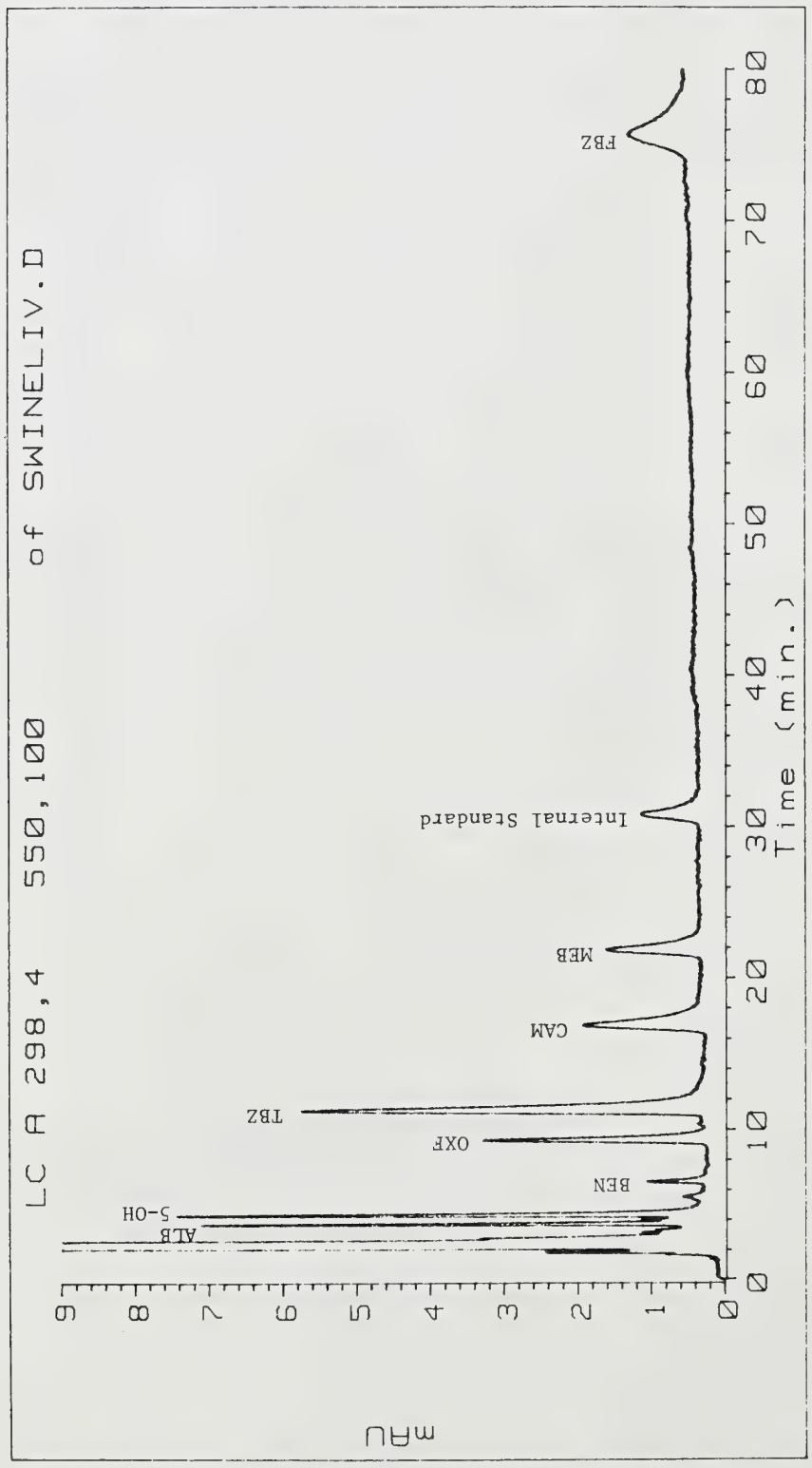
3. Sample Chromatograms



**100 ppb Benzimidazole External Standard
(200 ppb of Fenbendazole)**

I. DETERMINATIVE METHOD

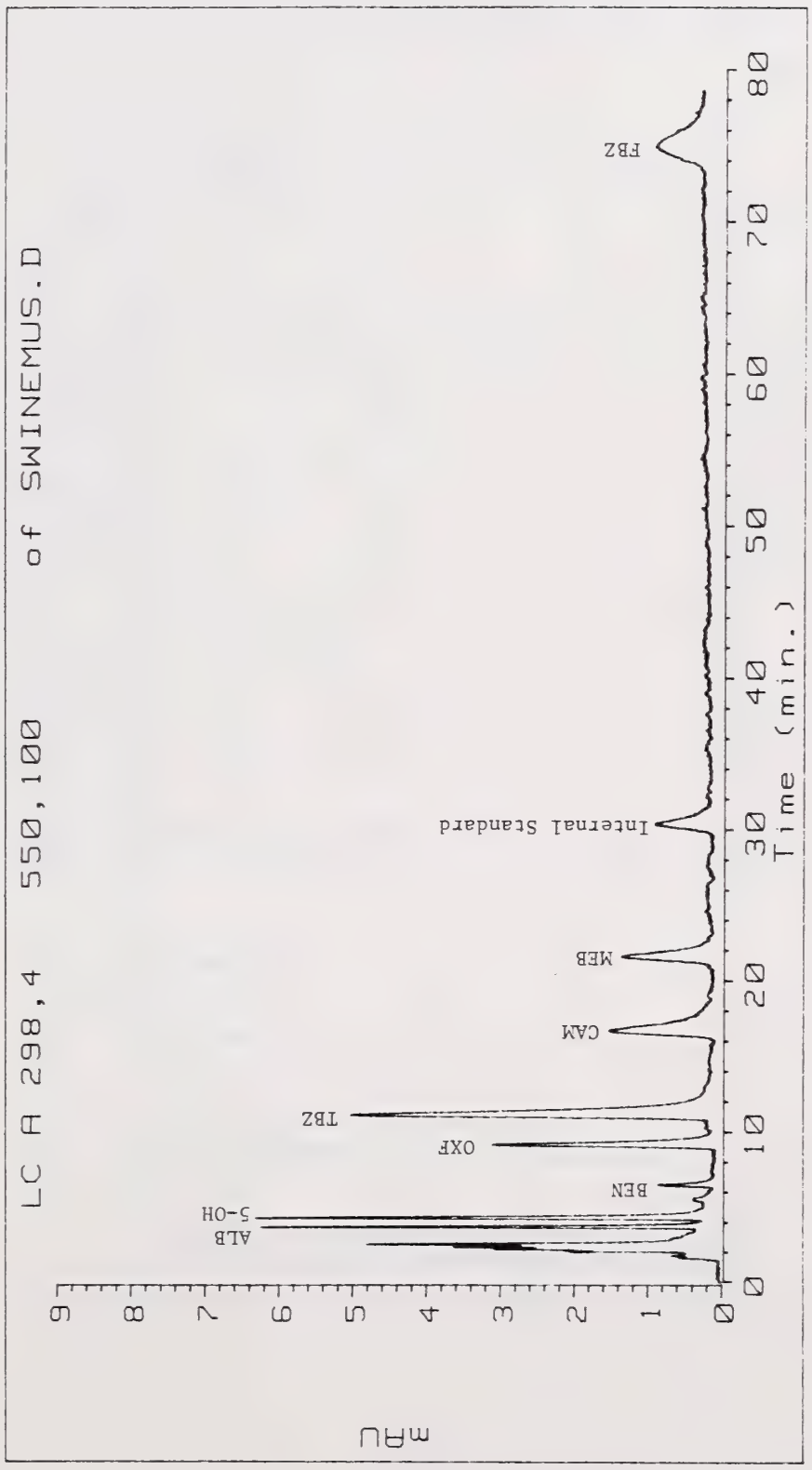
F. ANALYTICAL QUANTITATION (Continued)



100 ppb Swine Liver Recovery
(200 ppb of Fenbendazole)

I. DETERMINATIVE METHOD

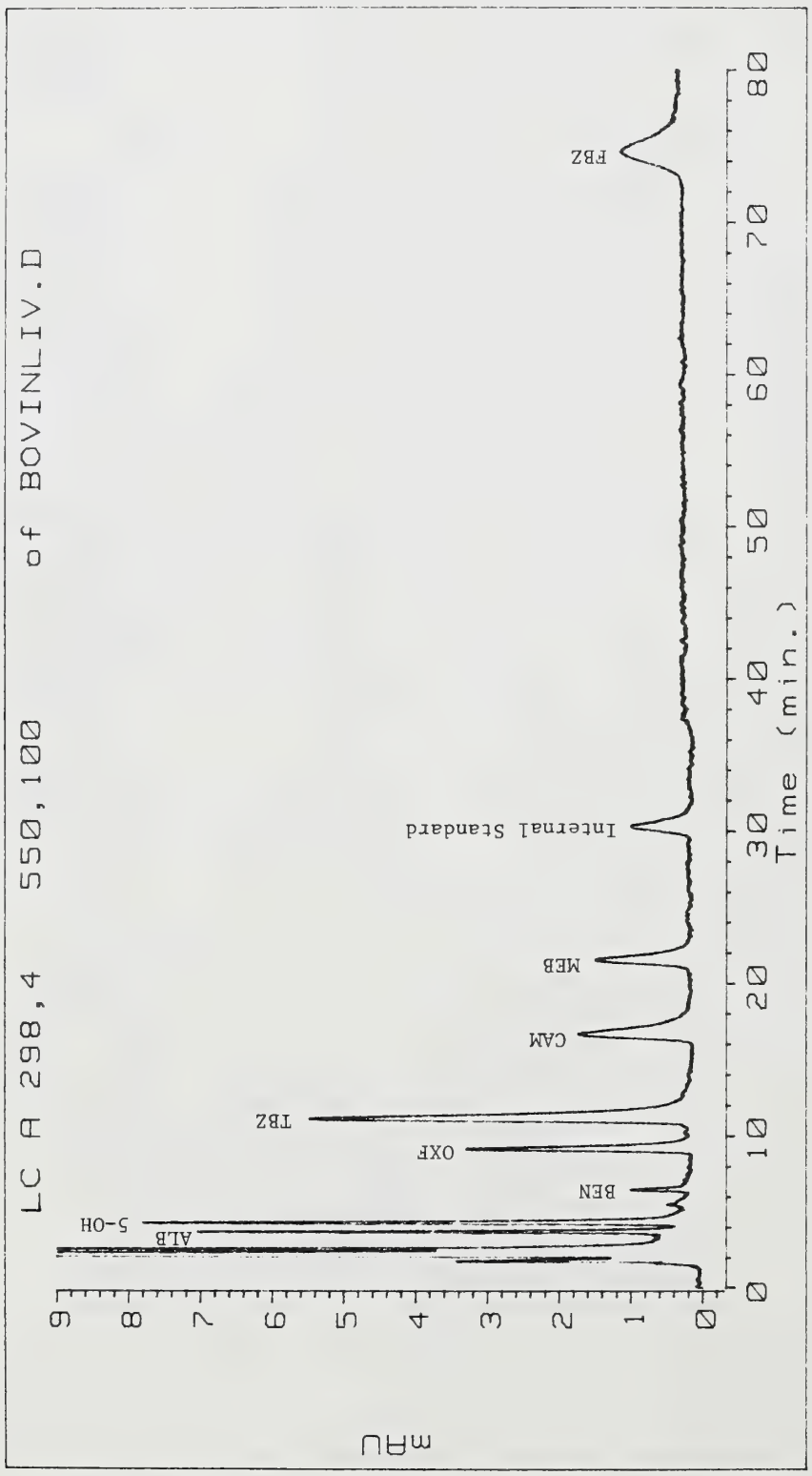
F. ANALYTICAL QUANTITATION (Continued)



**100 ppb Swine Muscle Recovery
(200 ppb of Fenbendazole)**

I. DETERMINATIVE METHOD

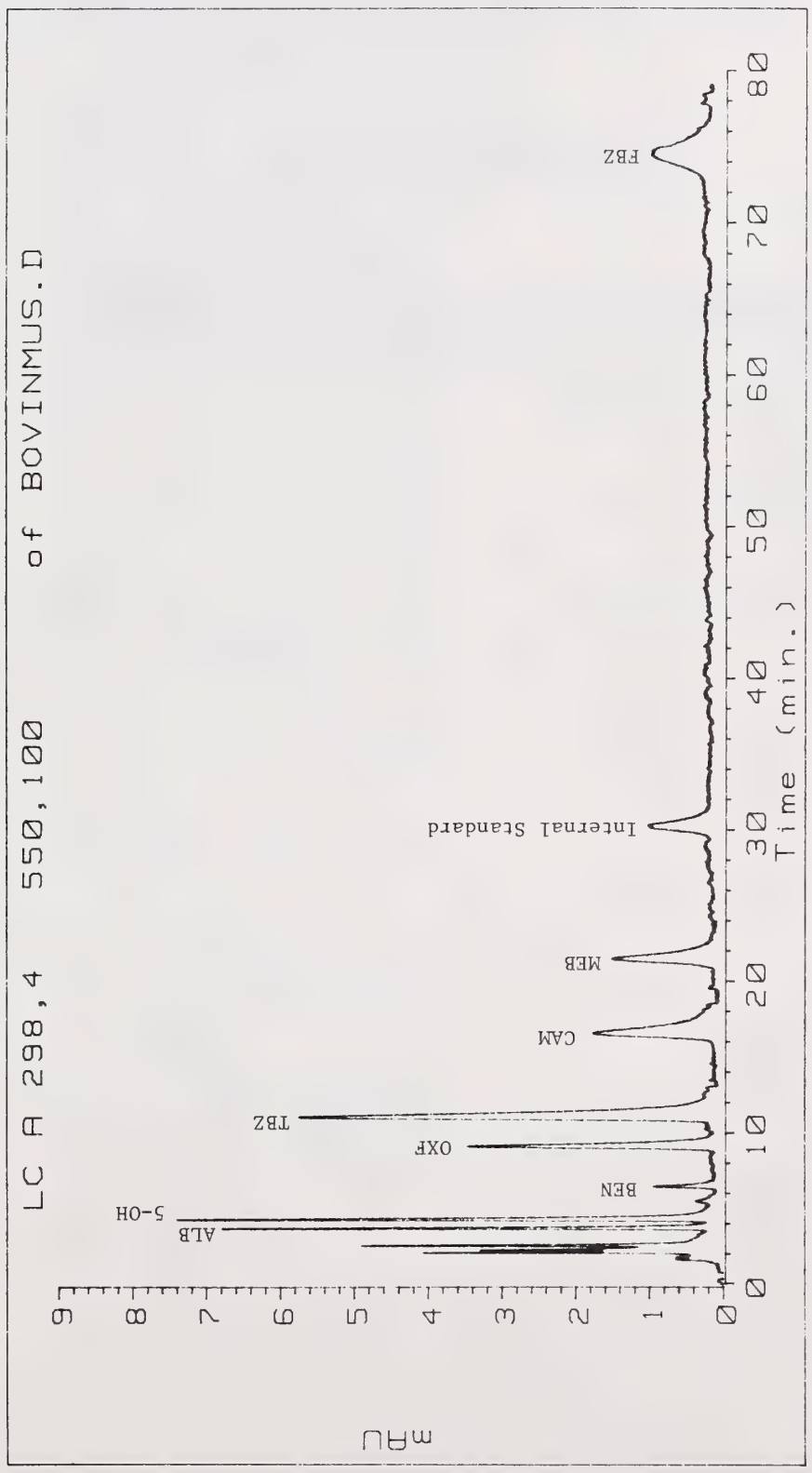
F. ANALYTICAL QUANTITATION (Continued)



100 ppb Bovine Liver Recovery
(200 ppb of Fenbendazole)

I. DETERMINATIVE METHOD

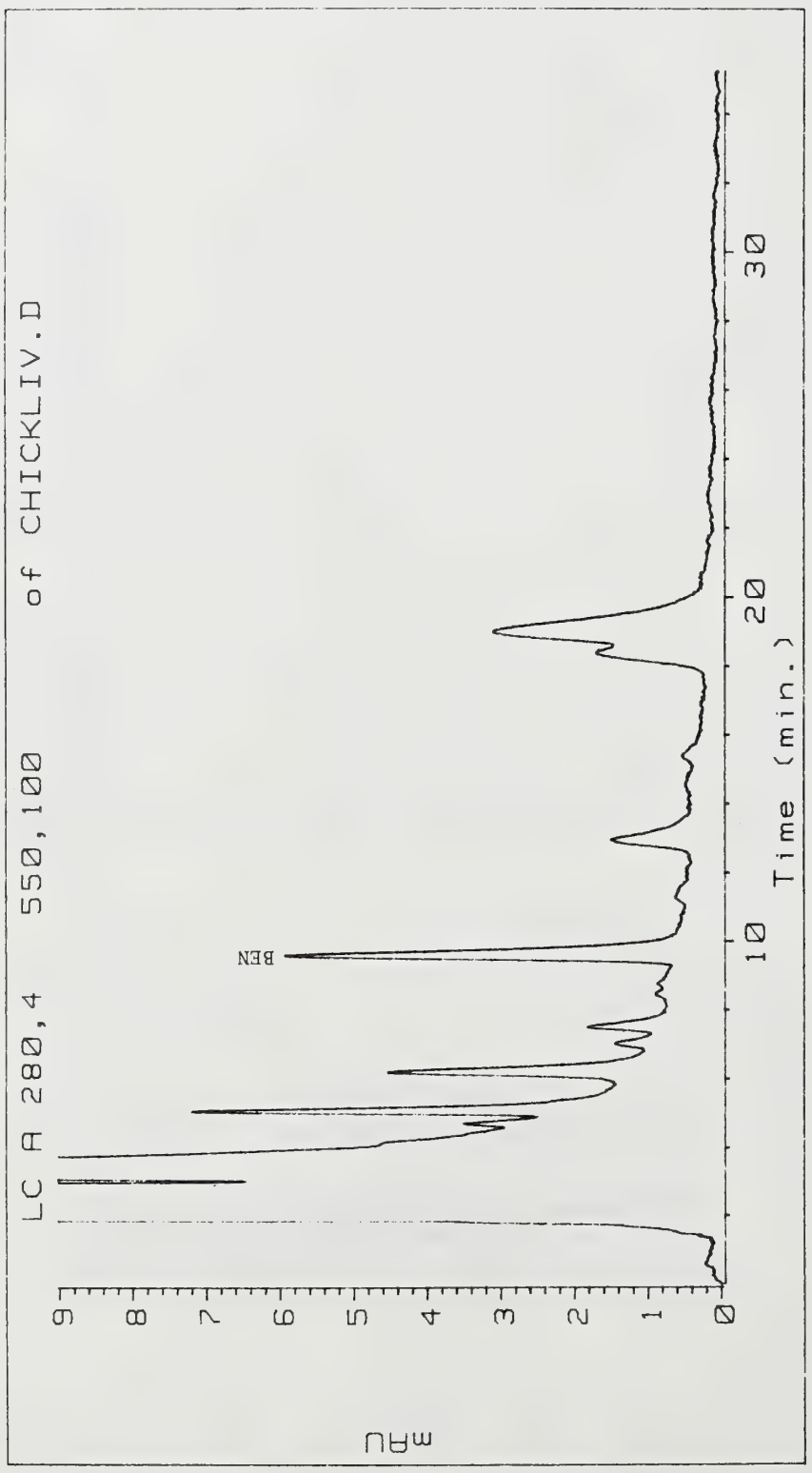
F. ANALYTICAL QUANTITATION (Continued)



**100 ppb Bovine Muscle Recovery
(200 ppb of Fenbendazole)**

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



100 ppb Benomyl Recovery from Chicken Liver

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Screen—Compare peak height of analyte of interest to that of internal standard to get an estimate of concentration.

b. Reanalysis of Positives—Determine the peak height for each component in the 0, 50, 100, and 200 ppb spiked samples that have been processed through the procedure. Using linear regression, construct a standard curve of benzimidazole concentration vs. peak height.

The equation is: $y = mx + b$

where x = peak height of benzimidazole
y = benzimidazole concentration
m = slope
b = y intercept

By use of the regression slope and intercept, compute the concentration (y) of the individual benzimidazole from its measured peak height.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Screen and Confirmation of Benzimidazole Residues in Animal Tissues

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Ethyl Acetate Reagent Alcohol Methanol Hexane Acetone	The organic reagents are very flammable. The vapors are extremely irritating to the skin, eyes, and respiratory system.	These solvents should only be used in an efficient fume hood, away from any heat-generating devices.
Dimethylsulfoxide	DMSO is an eye and skin irritant, has the capacity to dissolve and carry substances through skin.	Avoid skin contact; use plastic gloves when handling.
Triethylamine	Lachrymator. Corrosive, toxic, flammable liquid.	Use in efficient fume hood. Use plastic gloves when handling.
n-Butyl Iodide	Flammable liquid and irritant.	Use in efficient fume hood. Use plastic gloves when handling.
2-Mercaptobenzi-midazole	Toxic irritant.	Use in efficient fume hood. Use plastic gloves when handling.
MTBSTFA	Irritating to respiratory system and skin.	Use in efficient fume hood. Use plastic gloves when handling.
Trimethylanilinium	Flammable oxidizer. Toxic by inhalation, ingestion. Skin irritant.	Store tightly closed away from sources of ignition. Avoid contact with skin.

4. Disposal Procedures

Organic solvent mixture	See above.	Hold in waste containers until disposed of by contractor or in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Benzimidazoles*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

BENZIMIDAZOLES

Analyst: _____

Date Started: _____

Date Completed: _____

Sample weight 10 grams with final volume 1 mL unless noted otherwise.

Interset QC

Date of EtOH:0.2N HCl

_____

Not older than 14 days

	Sample Identification	Order of Addition of Na_2SO_4, K_2CO_3, EtOH	Response in nm								
			ALB	5-OH-TBZ	CAR (BEN)	OFZ	TBZ	CAM	MBZ	INT STD	FBZ
	% Recovery										
1	Recovery 100 ppb*										
2	External 100 ppb*										
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											

*FBZ Recovery and External @ 200 ppb

REMARKS:

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound List	*Acceptable Recovery %*	*Analytical Range (ppb)*	*Repeatability %CV*
2-(n-butylmercapto) benzimidazole	70-110[1]	200	< 20
Albendazole metabolite	70-110	50 to 200	< 20
5-Hydroxythiabendazole	70-110	50 to 200	< 20
Benomyl (Carbendazim)	70-110	50 to 200	< 20
Oxfendazole	70-110	50 to 200	< 20
Thiabendazole	70-110	50 to 200	< 20
Mebendazole	70-110	50 to 200	< 20
Cambendazole	70-110	50 to 200	< 20
Fenbendazole	70-110	100 to 800	< 20

[1]Determined values versus the known value.

2. Critical Control Points and Specifications

a. Intraset

Extraction procedure I.E.4. Order of addition of Na_2SO_4, K_2CO_3, and ethyl acetate.

b. Interset

Extraction procedure I.E.13. Ethanol:0.2N HCl solution must be prepared fresh every two weeks.

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards—Minimum criteria.

HPLC of standard curves containing all analytes at four levels: 0, 50, 100, and 200 ppb. FBZ at 0, 100, 200, and 400 ppb.

ii. Phase II: Self-Fortified Tissue Recoveries—Minimum criteria.

(a) Four levels—0, 50, 100, and 200 ppb. FBZ at 0, 100, 200, and 400 ppb.

(b) Swine liver and muscle; ovine liver and muscle; beef liver and muscle; chicken liver and muscle (carbendazim only); processed pork (canned ham).

(c) The number of replicates is at the discretion of the supervisor.

NOTE: Phases I and II may be performed concurrently. Submit summary data to Chemistry Division.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Phase III: Unknown Samples.

Analyze 16 samples—swine, processed pork, and chicken (carbendazim only)—from supervisor.

b. Acceptability criteria.

Refer to Section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least 1 check sample biweekly per analyst containing at least one of the benzimidazoles and using different species. At least 1 of 4 of positive samples is to be confirmed by mass spectrometry.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on difference between replicates.

(c) All percent recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver, muscle, processed products.

b. Sample receipt size: 50 g.

c. Condition upon receipt: Cold (less than 10° C).

d. Sample storage:

i. Time: 1 month.

ii. Condition: Away from light, −20° C.

I. DETERMINATIVE METHOD

F. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	a. Screen: Fortified blank (100 ppb, except FBZ at 200 ppb) and samples. Species/tissues can be mixed for screen. b. Reanalysis: Blank tissue and fortified blanks, in the appropriate species/tissues, at three concentrations (50, 100, and 200 ppb, except FBZ at 100, 200, and 400 ppb) to generate data for standard curve.
7. Sensitivity	a. Lowest detectable level (LDL): 30 ppb. b. Lowest reliable quantitation (LRQ): 50 ppb.

II. CONFIRMATORY METHOD

A. INTRODUCTION

The confirmation of benzimidazoles relies upon four factors. First, the analyte UV spectrum is determined and matched with standard material. Second, the analytes are derivatized using tertiary butyldimethylsilyl amine (TBDMS). Thirdly, the derivatized analytes are separated using gas chromatography instead of HPLC, which was used in the determinative method. Lastly, the analytes are detected using selected ion electron impact mass spectrometry. Refer to the Determinative Method, Section A, for additional information.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.1.

2. Instrumentation

a. GC/MS HP 5890/5970B, or equivalent, capable of electron ionization, selected ion monitoring.

b. Column: 12-15 meter × 0.2 mm i.d. fused silica crosslinked methyl silicone with film thickness 0.33 micron, Hewlett Packard #19091A-101, or equivalent.

c. Photodiode detector Array Deter, Hewlett-Packard 1090 or equivalent equipped with a solvent delivery and separations system equivalent to that used in I. F. 1.

C. REAGENTS

Reagent List

a. Trimethylphenylammonium hydroxide, BARB-PRER (Alltech Associates).

b. N-methyl-N-(tert-Butyldimethylsilyl) trifluoroacetamide, MTBSTFA (Pierce).

c. Perfluorotributylamine (PFTBA).

d. Also refer to Determinative Method, section C.

II. CONFIRMATORY METHOD

D. STANDARDS

1. Source

a. See Determinative Method, section D.1, for source of standards.

b. See section E below for directions on how to synthesize TBDMS derivatives of standards.

2. Standards

a. Use mixed standards as prepared for Determinative Method, section D.2, and synthesize TBDMS derivatives following directions given in section E below.

b. Approximately 3 μL of mixed standard solution (containing about 40 ng of each standard) will be injected into the GC/MS.

Prior to submission for MS analysis, determine the analyte UV spectrum. If the spectrum matches that for standard material, proceed to E.

E. SAMPLE PREPARATION AND CLEANUP

1. Derivatization

a. Transfer the remaining extract from the HPLC screening procedure to a 10 mL disposable glass centrifuge tube and evaporate to dryness on heating block at 40° C under N_2.

b. Add 0.5 mL 2N HCl and heat at 110° C for 1 hour.

c. Cool and add 1.0 mL H_2O and neutralize with solid K_2CO_3 by adding 50-100 mg portions until effervescence ceases. Add a slight excess.

d. Extract 3 times with 2 mL ethyl acetate, shaking each time for 2 minutes and transferring the organic solvent to a 10 mL disposable glass centrifuge tube.

e. Evaporate the combined ethyl acetate fractions to dryness in a heating block at 50° C under N_2.

f. Add 50 μL of N-(methyl)-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and heat in a 105° C heating block for 2 hours.

g. Cool and inject 2 μL into the GC/MS.

For samples suspected of containing cambendazole, an alternate confirmation is necessary.

a1. Transfer the remaining extract from the HPLC screen and evaporate to dryness as in E.1.a above.

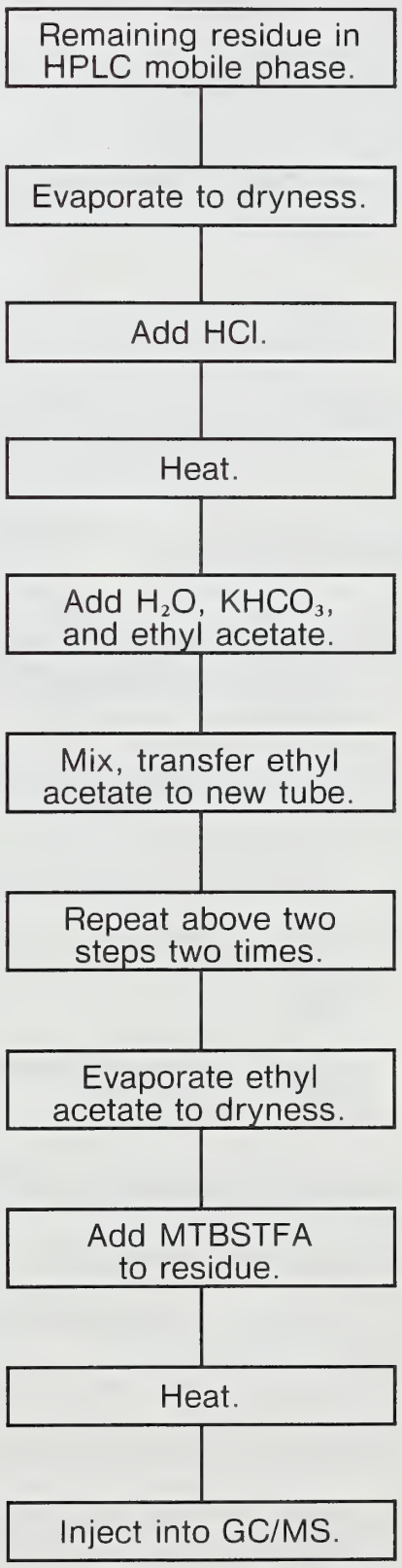
b1. Dissolve the residue in 50 μL of 0.2N trimethylphenylammonium hydroxide in methanol and inject 3 μL into the GC/MS under the same conditions as above.

NOTE: Two peaks will be observed, both having identical mass spectra and exhibiting retention time difference of about 10-15 seconds under the stated conditions. Presumably these are structural isomers due to the methylation of the carbamate functional group. Both must be present for confirmation.

II. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP (Continued)

2. Flow Chart Summary



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions.

Injection temperature: 260° C.
Initial temperature: 150° C.
Initial hold: 2 minutes.
Programmed 6° C/minute.
Final temperature: 300° C.
Run time: 20 minutes.
Equilibration time: 0.5 minutes.
Purge-off time: 1.5 minutes.

b. MS conditions.

Interface: 300° C.
Dwell time: 100 milliseconds.
Calibration standard: PFTBA used in autotune mode.

2. Required Samples for GC/MS Analysis

Prior to analyzing TBDMS derivatives of samples, perform an autotune followed by an injection of approximately 40 ng of each of the TBDMS benzimidazoles in a combined mixed standard. Obtain at least three scans across each peak and average the masses at each ion used for identification to obtain the peak centroid for each.

Set up a retention time window about each compound and monitor its ions within that window; then switch to the ions for the next compound until all compounds are monitored. The width of the retention time window should be the time from valley to valley in the chromatogram of the standard mixture.

3. Criteria for Confirmation

a. Successful confirmation requires the following:

i. All compounds observed at the same retention time as the reference material (standard).

ii. UV spectrum matches that for standard material.

iii. All ions must be present.

iv. Ion ratios must be within 15% of ion ratios for standard material.

Some of the ions monitored, those representing loss of derivatizing reagent, are not structurally significant. They are indicated below by an asterisk.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

b. Retention time specifications.

Compound	*Relative Retention Time[1] (min)*
2-(n-butylmercapto) benzimidazole Internal Standard	1.00
Benomyl (Carbendazim)	1.01
Thiabendazole	1.09
5-OH thiabendazole	1.49
2-Aminosulfone albendazole	1.68
Fenbendazole	1.71
Mebendazole	1.89
Oxfendazole	2.25
Cambendazole	1.28 1.29

[1]Relative to internal standard.

c. Specific ions required.

Compound	*Ions Monitored*
2-(n-butylmercapto) benzimidazole Internal Standard	320, 263*,207*
Benomyl (Carbendazim)	361, 304*, 248*
Thiabendazole	315*, 300*, 258
5-OH thiabendazole	445, 430*, 388*
2-Aminosulfone albendazole	467, 410*, 354*
Fenbendazole	469, 412*, 356*
Mebendazole	465, 408*, 352*
Oxfendazole	485, 469, 428*, 372*
Cambendazole	330, 288, 243

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

i. Ion ratios.

Compound	*Ions*	*Expected Ratio*
Carbendazim	361/248	0.138
	304/248	1.026
Thiabendazole	315/258	0.060
	300/258	0.026
5-Hydroxy thiabendazole	445/388	0.163
	430/388	0.036
2-aminosulfone albendazole	467/354	0.056
	410/354	0.794
Fenbendazole	469/356	0.288
	412/356	0.832
Mebendazole	465/352	0.071
	408/352	0.900
Oxfendazole	485/372	0.144
	428/372	0.690
Cambendazole	330/288	0.752
	330/243	0.829
	288/243	1.102

ii. Postulated structures of confirming ions. Refer to BNZ-39 through BNZ-46.

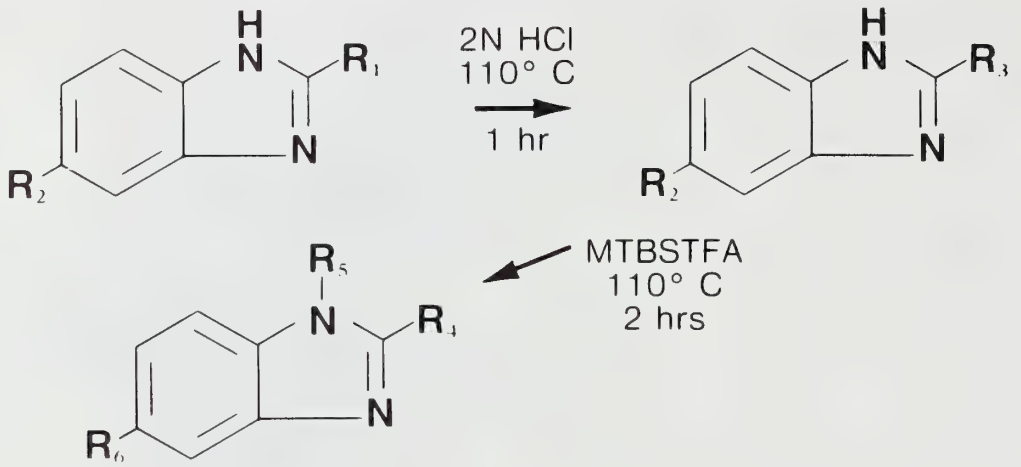
4. Interferences

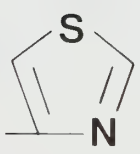
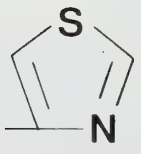
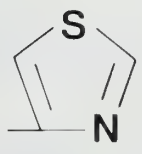
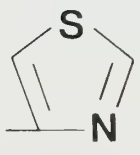
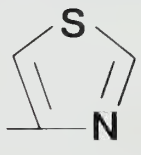
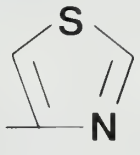
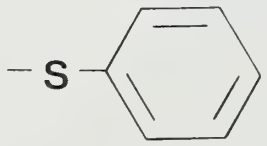
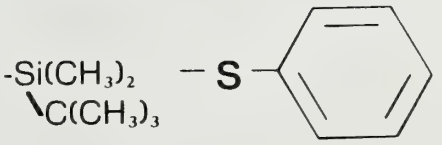
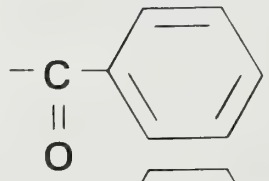
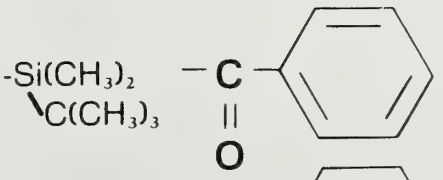
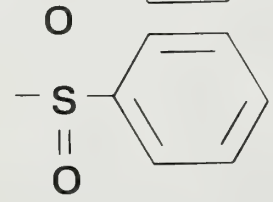
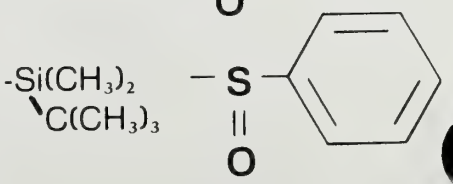
No known interferences at this time.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Table 1 Hydrolysis and Derivatization Reactions for Eight Benzimidazoles.

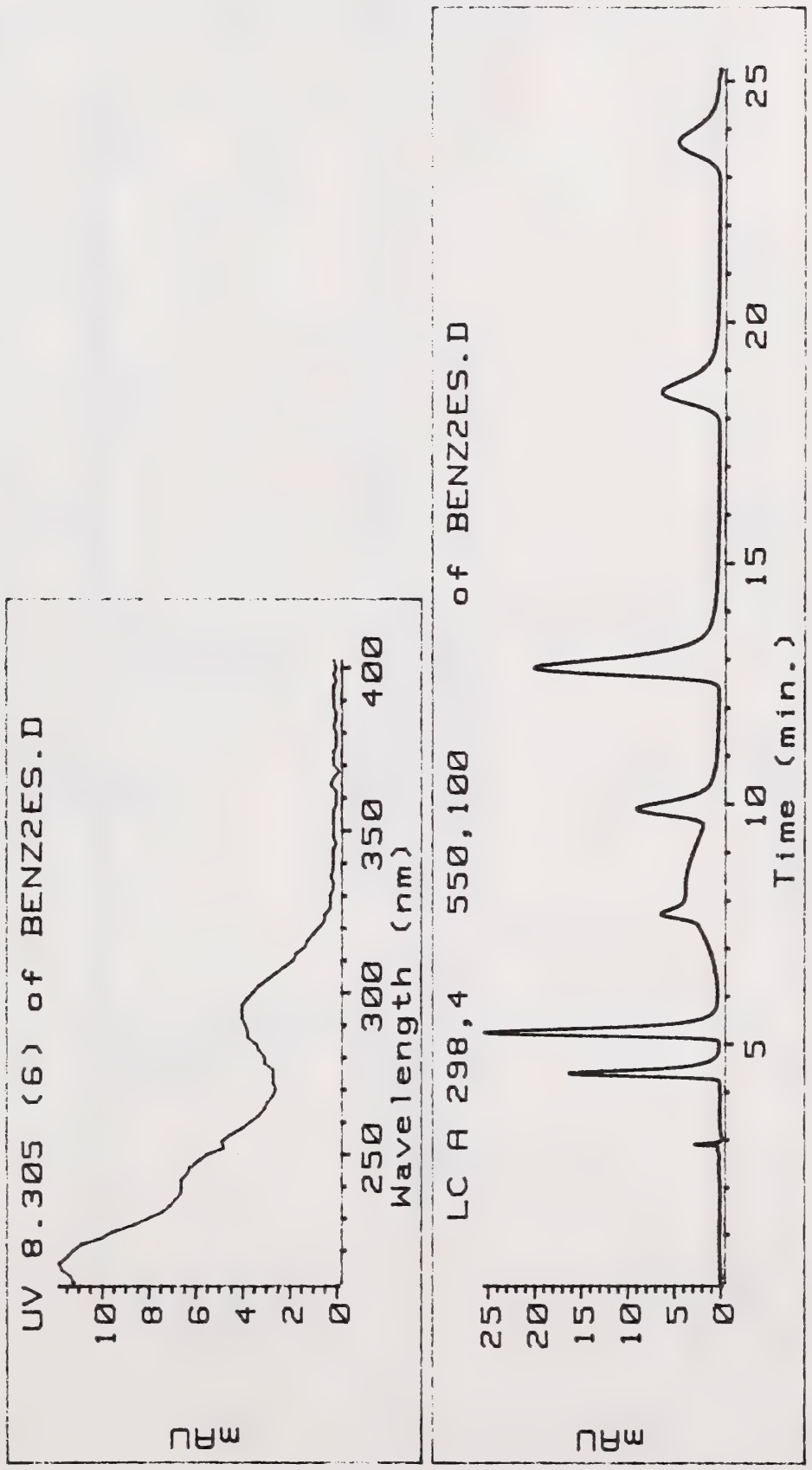


Compound	*R_1*	*R_2*	*R_3*	*R_4*	*R_5*	*R6*
I. S.	$-S(CH_2)_3CH_3$	-H	$-S(CH_2)_3CH_3$	$-S(CH_2)_3CH_3$	$-Si(CH_3)_2C(CH_3)_3$	-H
BEN (CAR)	$-NHCOOCH_3$	-H	$-NH_2$	$-NHSi(CH_3)_2C(CH_3)_3$	$-Si(CH_3)_2C(CH_3)_3$	-H
TBZ		-H			$-Si(CH_3)_2C(CH_3)_3$	-H
5-OH		-OH			$-Si(CH_3)_2C(CH_3)_3$	$-O-Si(CH_3)_2C(CH_3)_3$
ALB (aminosulfone)	$-NH_2$	$-SO_2(CH_2)_2CH_3$	$-NH_2$	$-NHSi(CH_3)_2C(CH_3)_3$	$-Si(CH_3)_2C(CH_3)_3$	$-SO_2(CH_2)_2CH_3$
FBZ	$-NHCOOCH_3$		$-NH_2$	$-NHSi(CH_3)_2C(CH_3)_3$	$-Si(CH_3)_2C(CH_3)_3$	
MBZ	$-NHCOOCH_3$		$-NH_2$	$-NHSi(CH_3)_2C(CH_3)_3$	$-Si(CH_3)_2C(CH_3)_3$	
OFZ	$-NHCOOCH_3$		$-NH_2$	$-NHSi(CH_3)_2C(CH_3)_3$	$-Si(CH_3)_2C(CH_3)_3$	

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

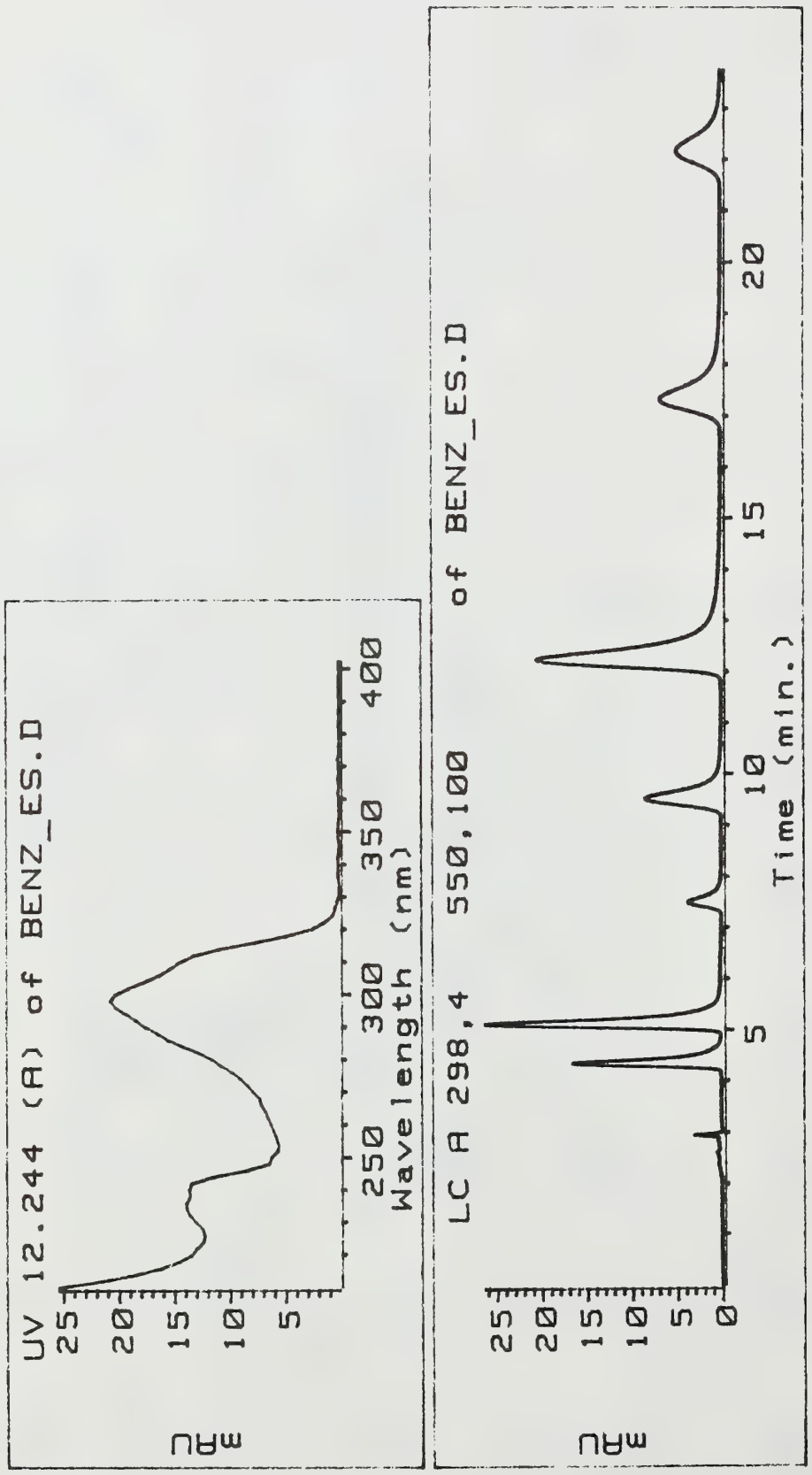
5. Chromatograms
   UV Spectra



FENBENDAZOLE

II. CONFIRMATORY METHOD

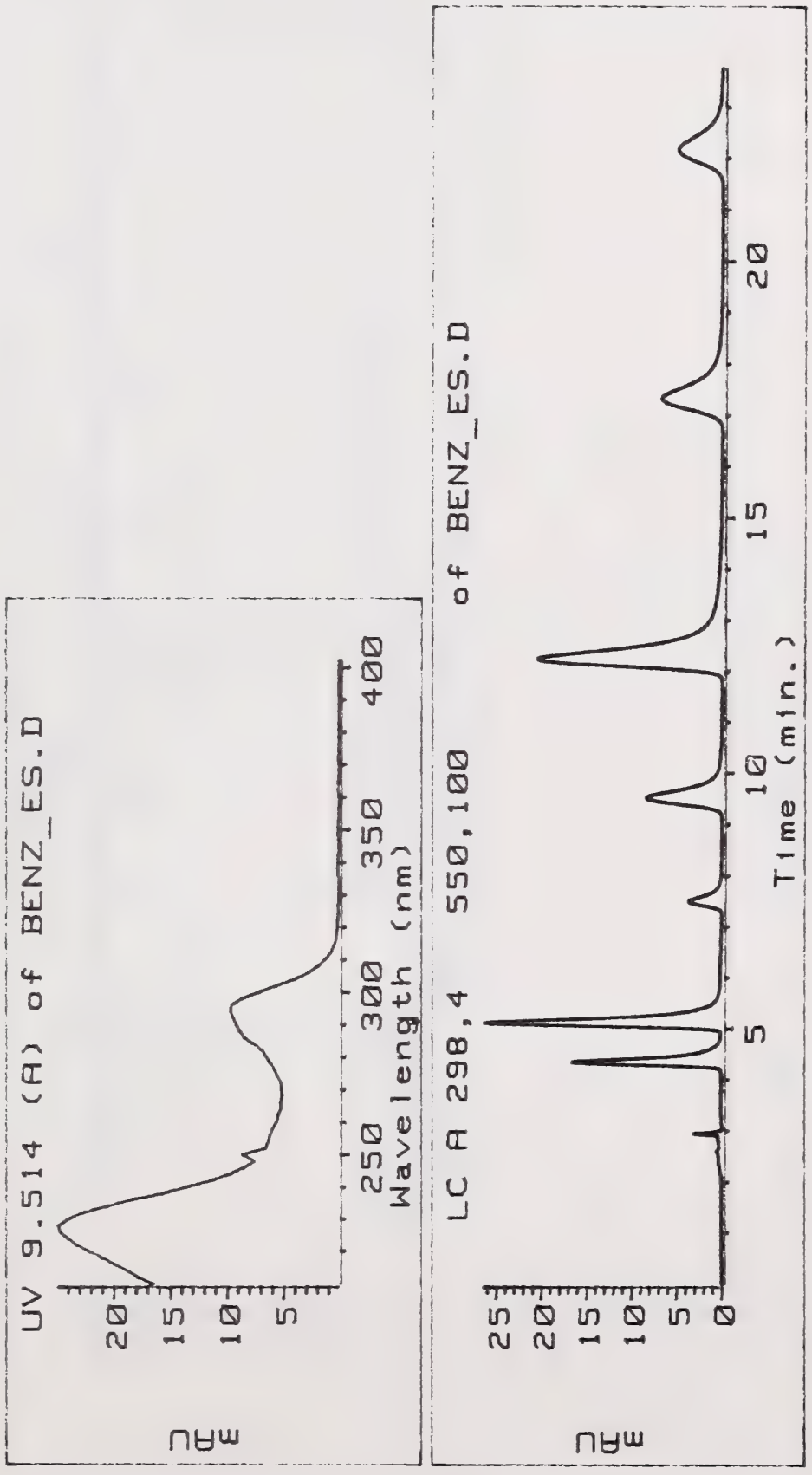
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



THIABENDAZOLE

II. CONFIRMATORY METHOD

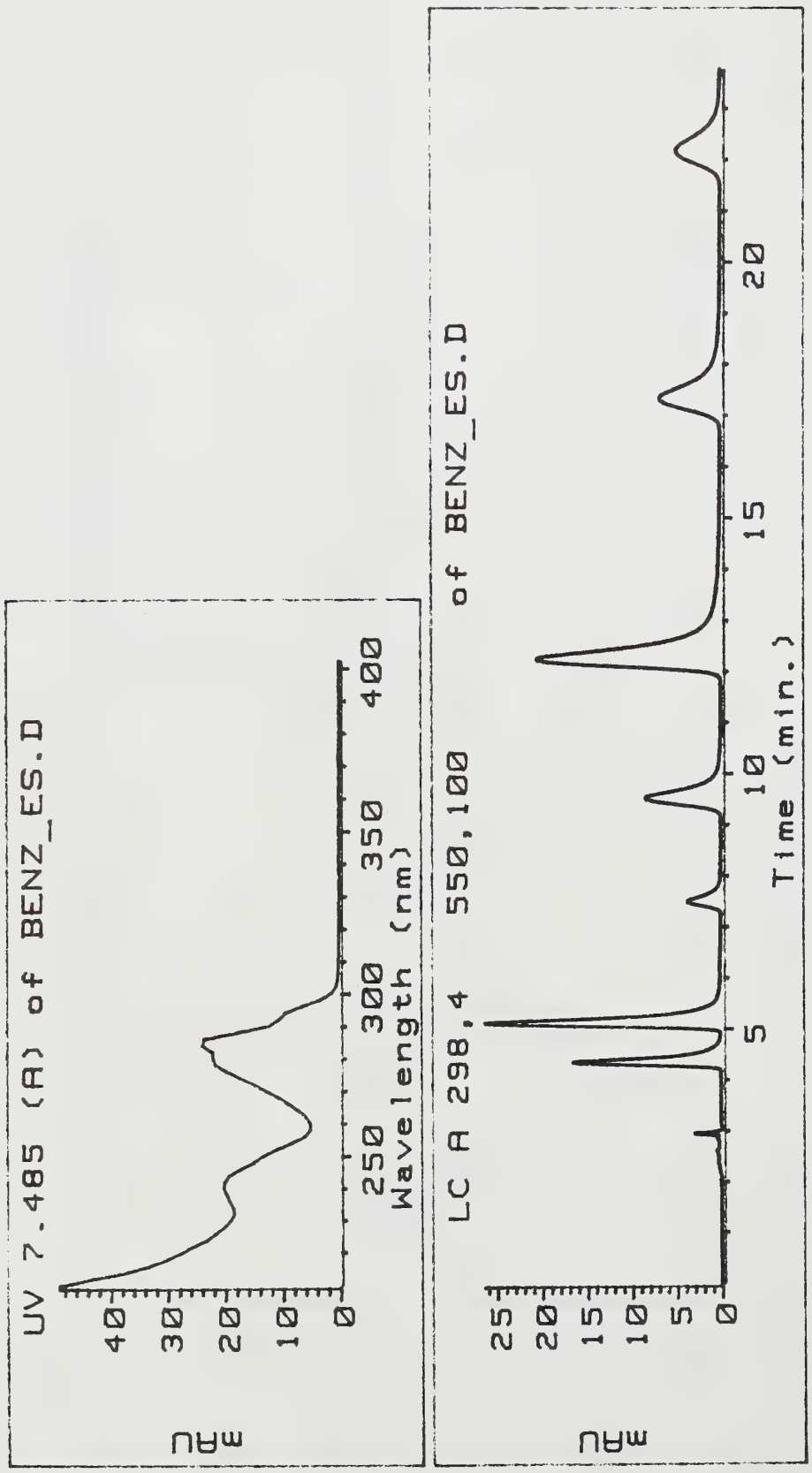
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



OXFENDAZOLE

II. CONFIRMATORY METHOD

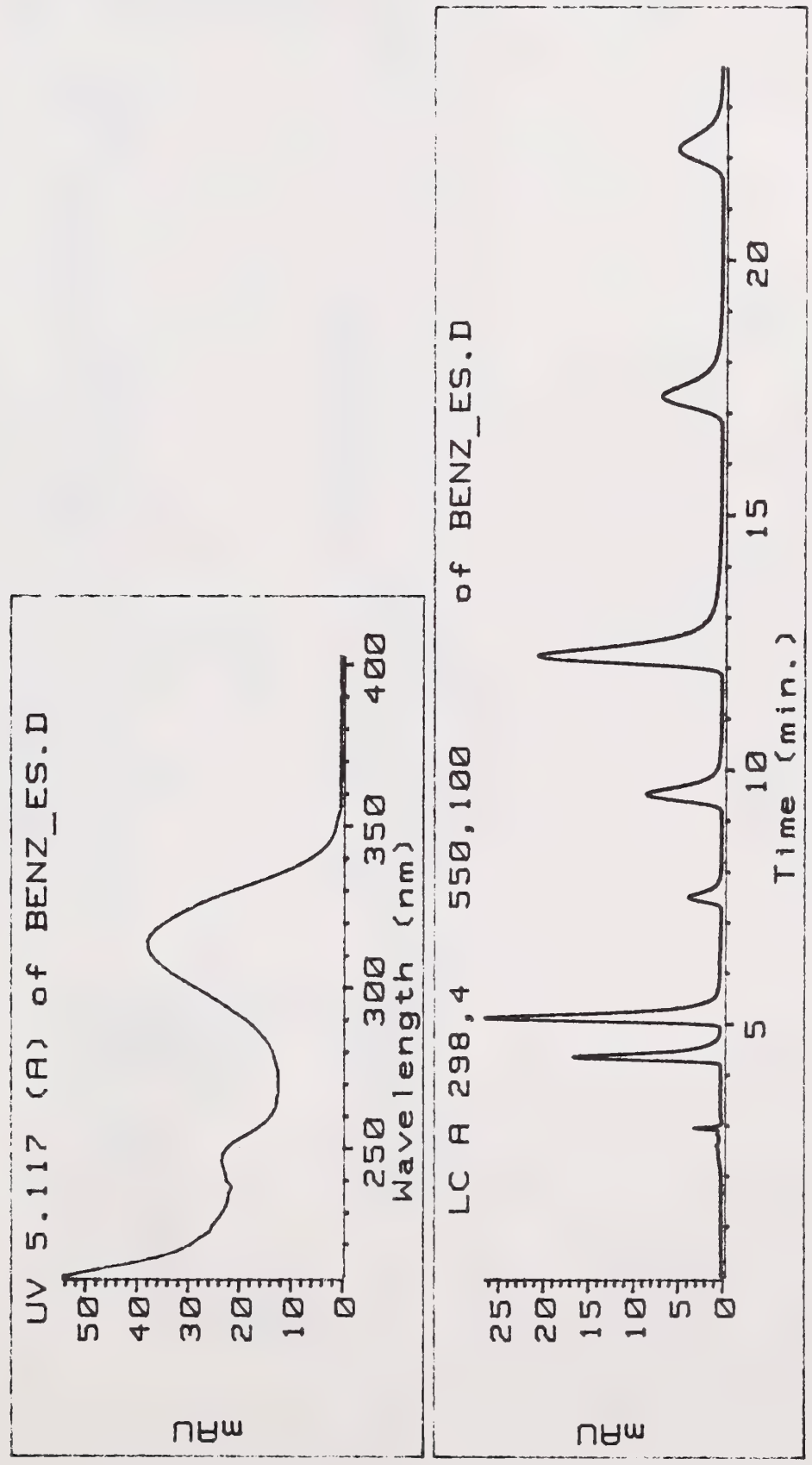
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



BENOMYL

II. CONFIRMATORY METHOD

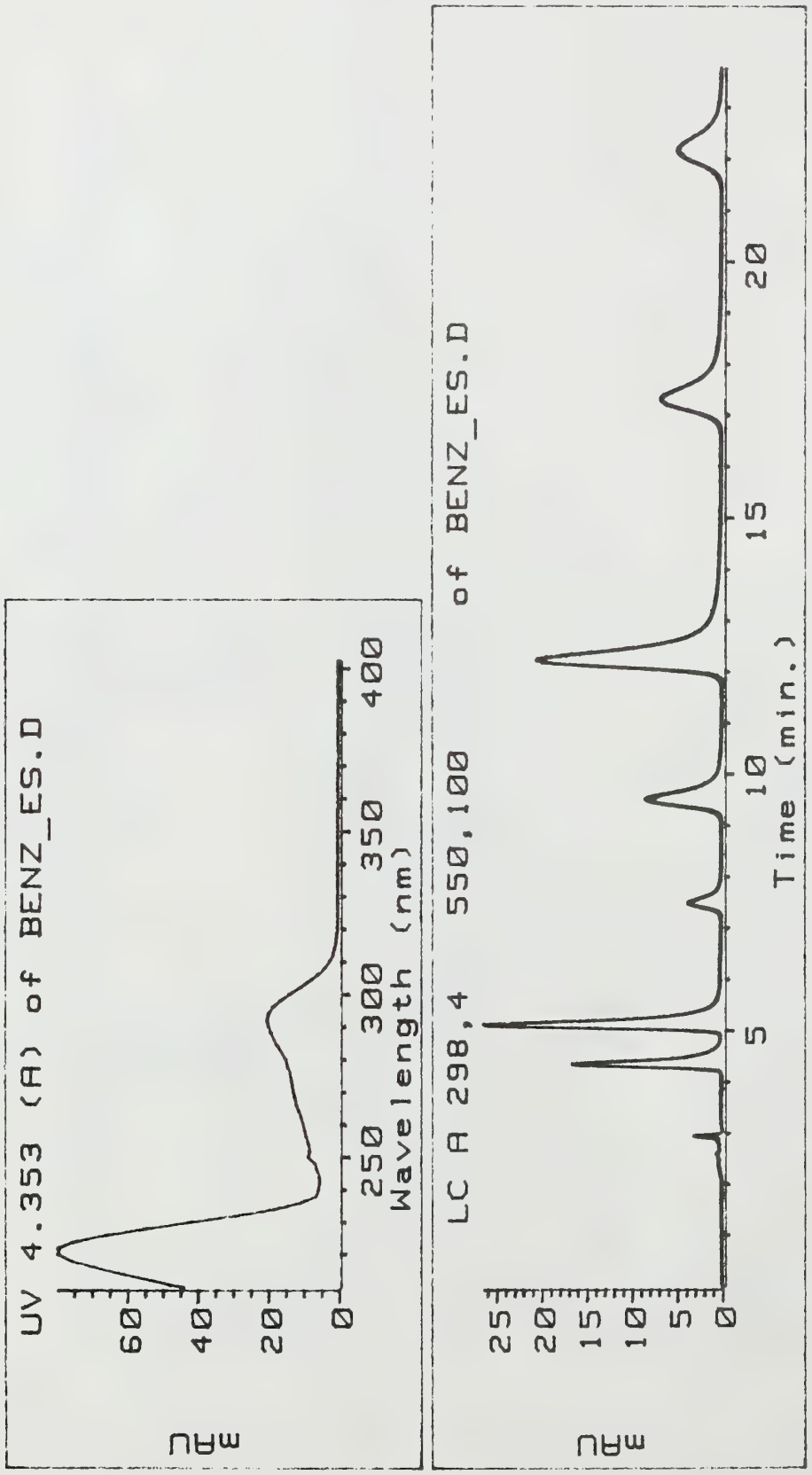
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



5-HYDROXYTHIABENDAZOLE

II. CONFIRMATORY METHOD

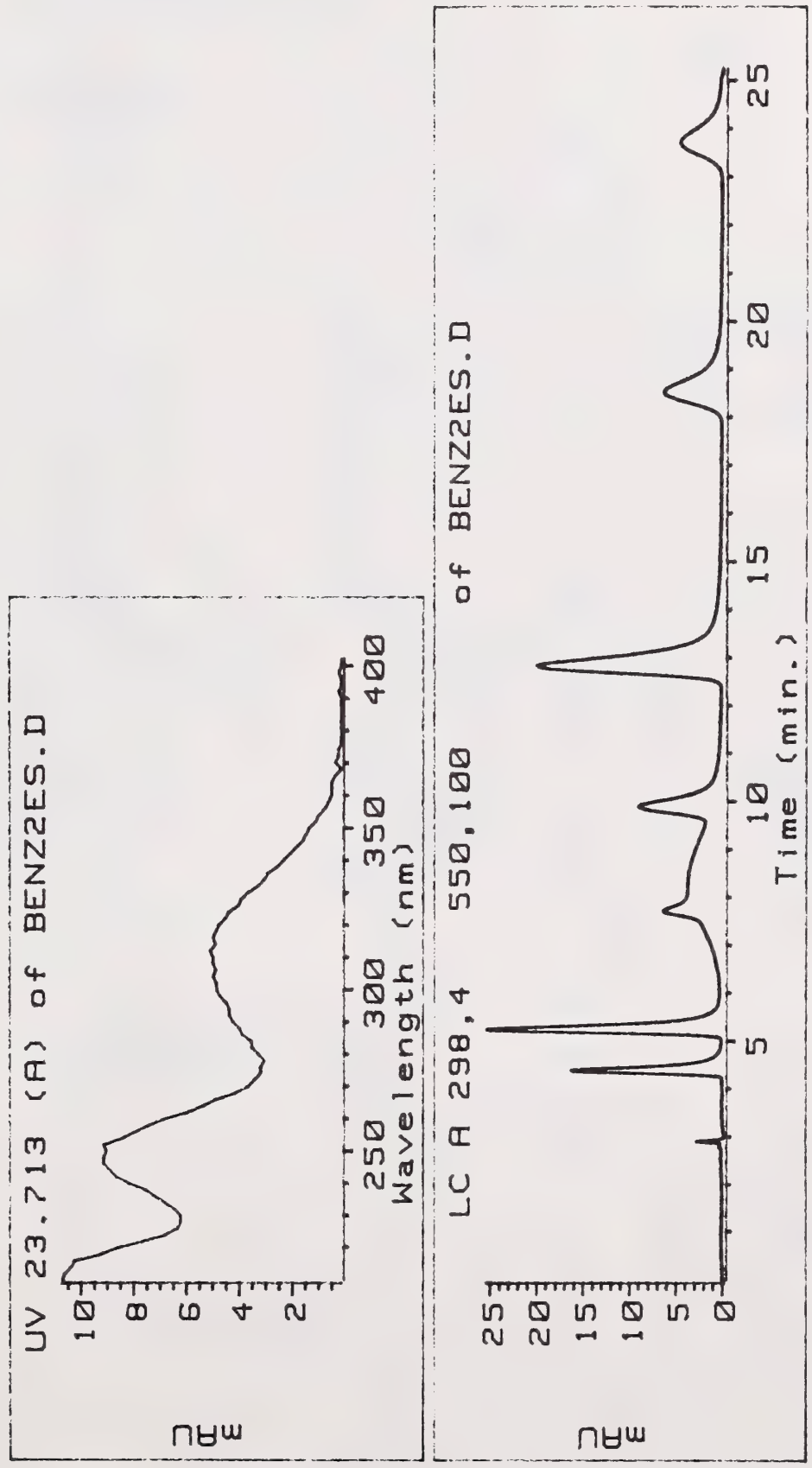
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



ALBENDAZOLE

II. CONFIRMATORY METHOD

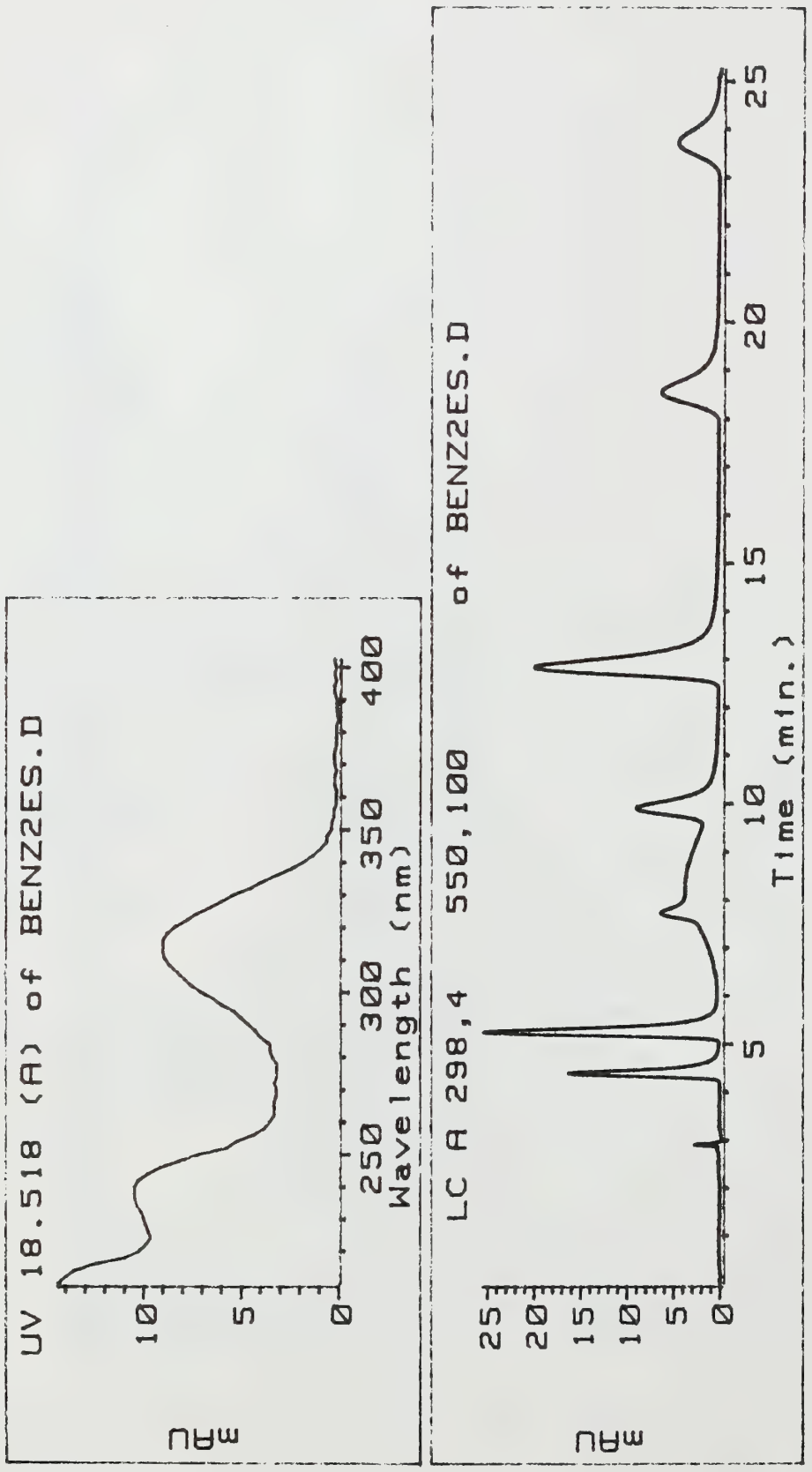
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



MEBENDAZOLE

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



CAMBENDAZOLE

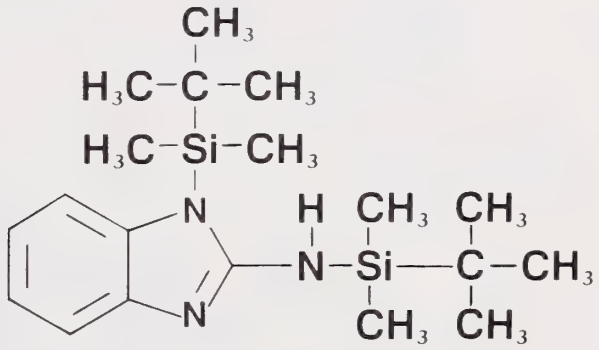
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

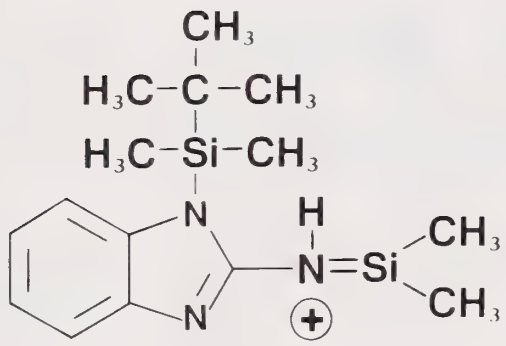
Postulated Structures of Carbendazim (BEN) Derivatives Used for MS Confirmation

MASS

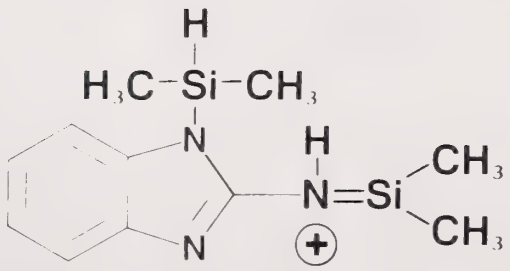
361



304



248



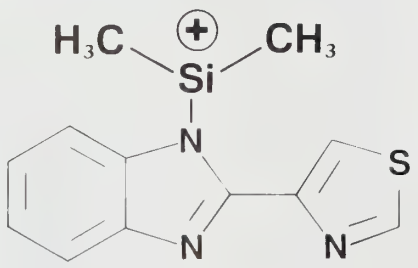
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

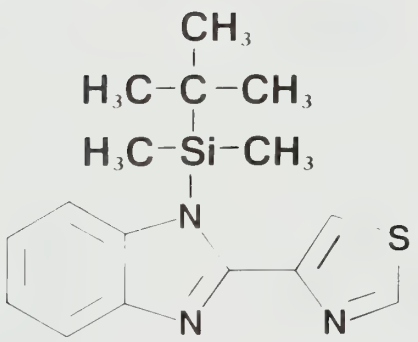
Postulated Structures of Thiabendazole Derivatives Used for MS Confirmation

MASS

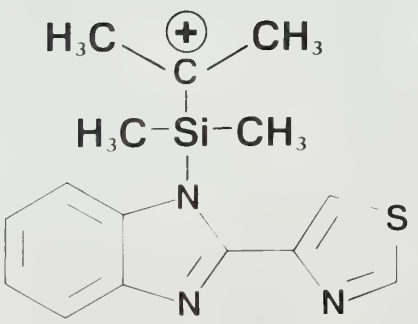
258



315



300



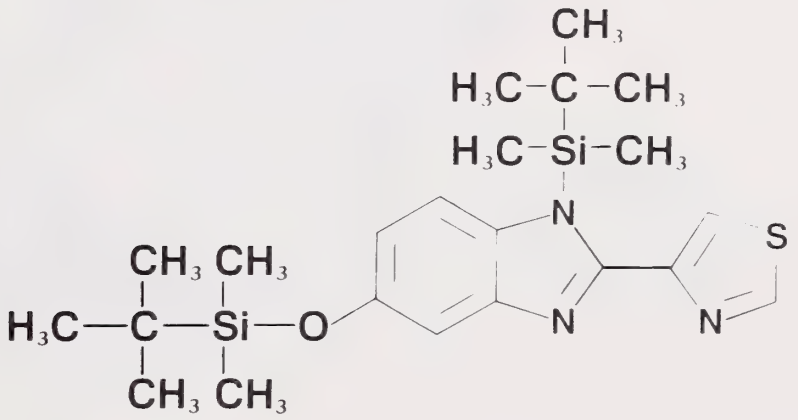
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

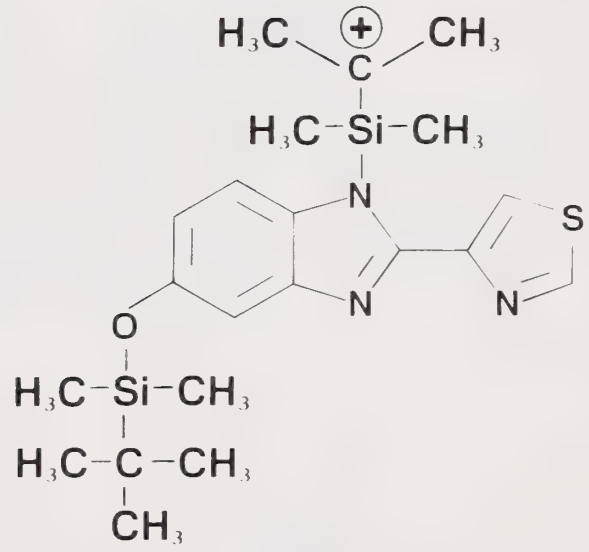
Postulated Structures of 5-Hydroxy Thiabendazole Derivatives Used for MS Confirmation

MASS

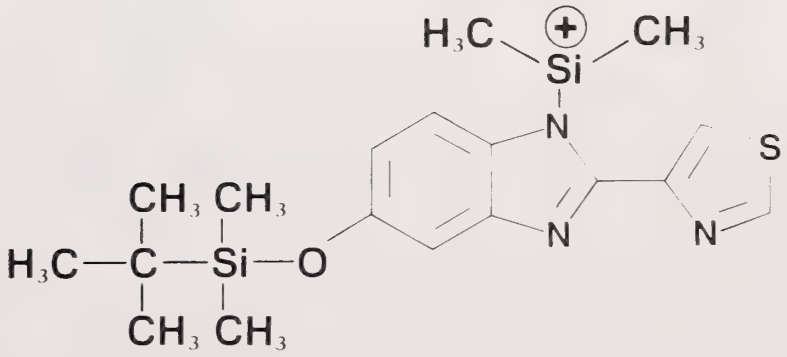
445



430



388



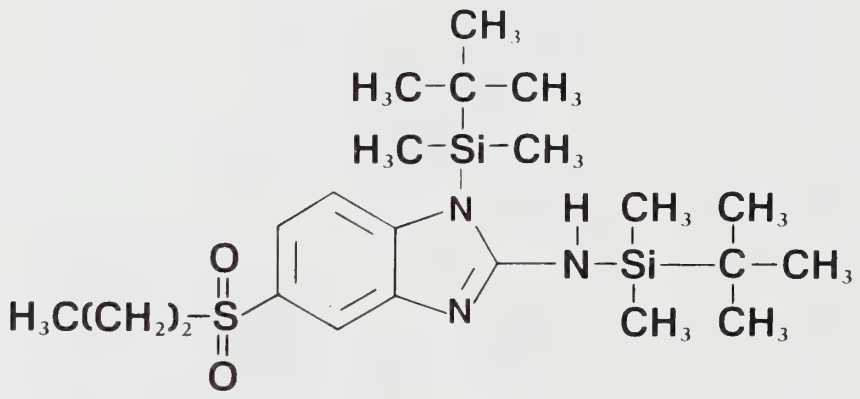
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

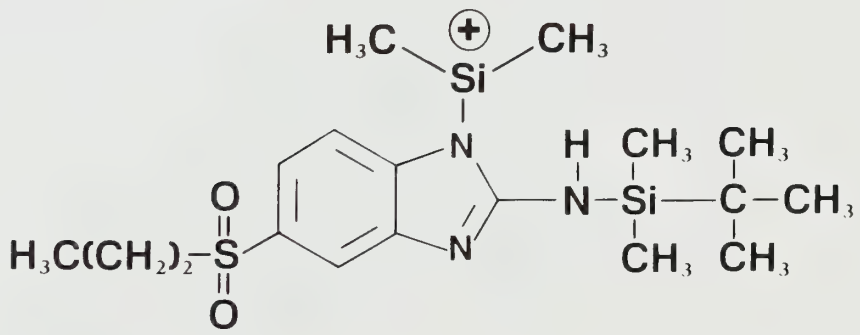
Postulated Structures of 2-Aminosulfone Albendazole Derivatives Used for MS Confirmation

MASS

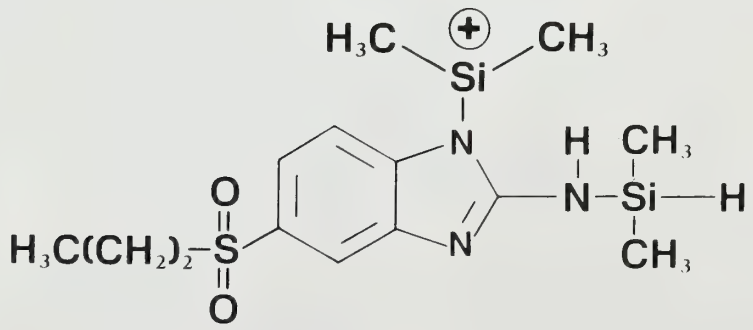
467



410



354



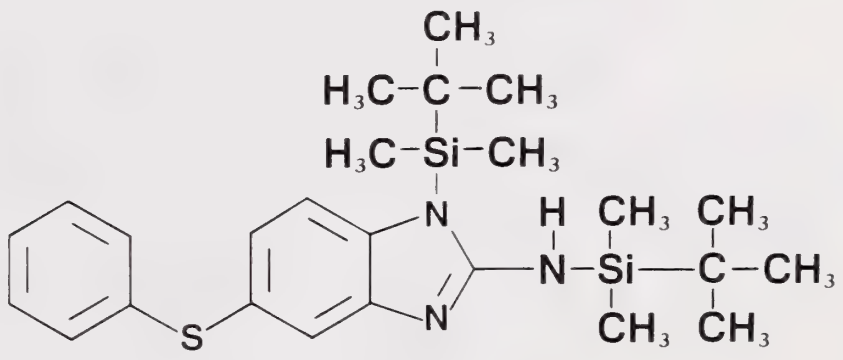
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

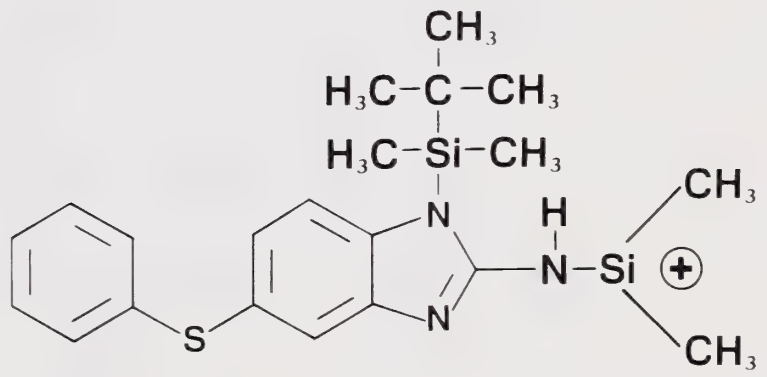
Postulated Structures of Fenbendazole Derivatives Used for MS Confirmation

MASS

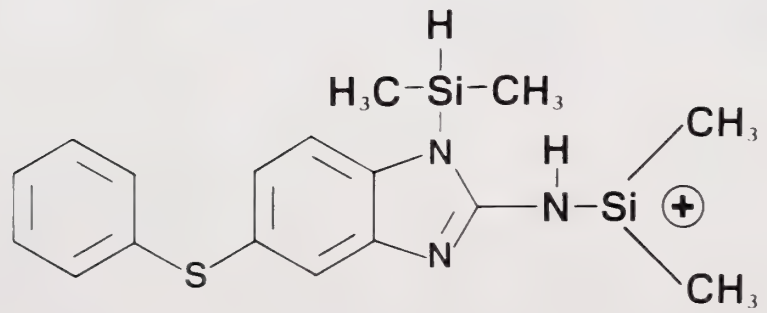
469



412



356



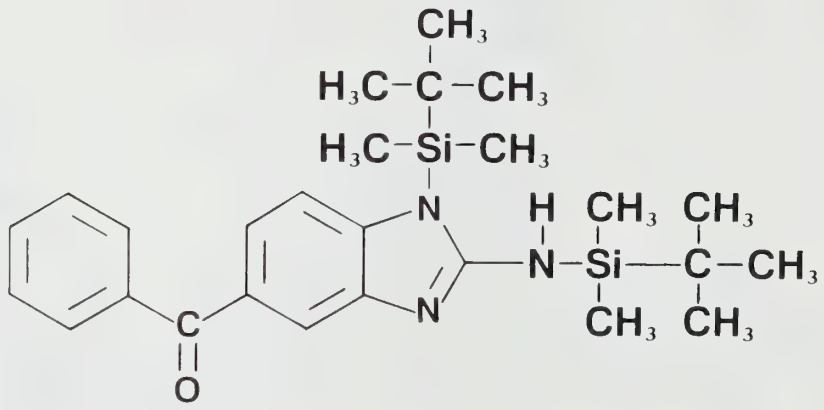
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

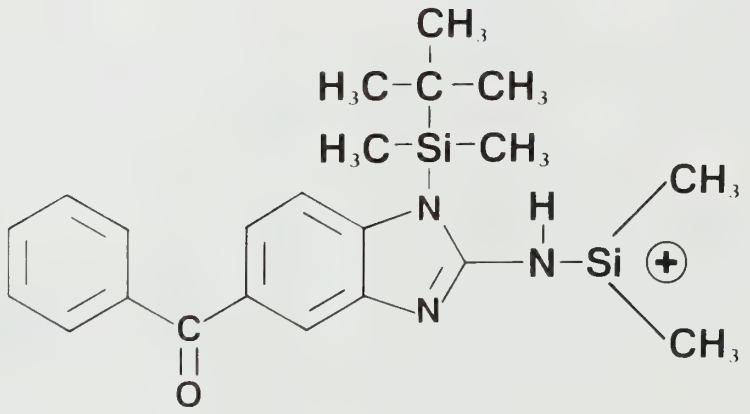
Postulated Structures of Mebendazole Derivatives Used for MS Confirmation

MASS

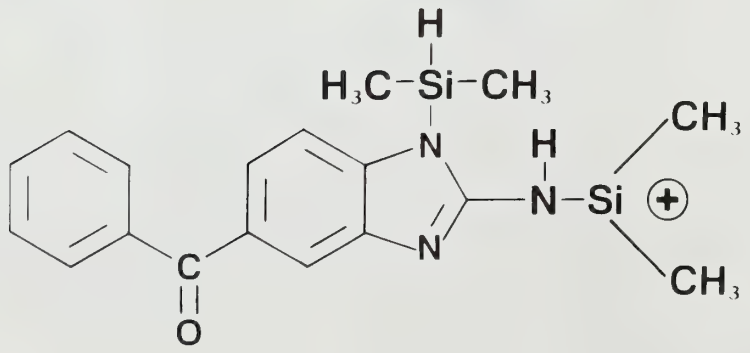
465



408



352



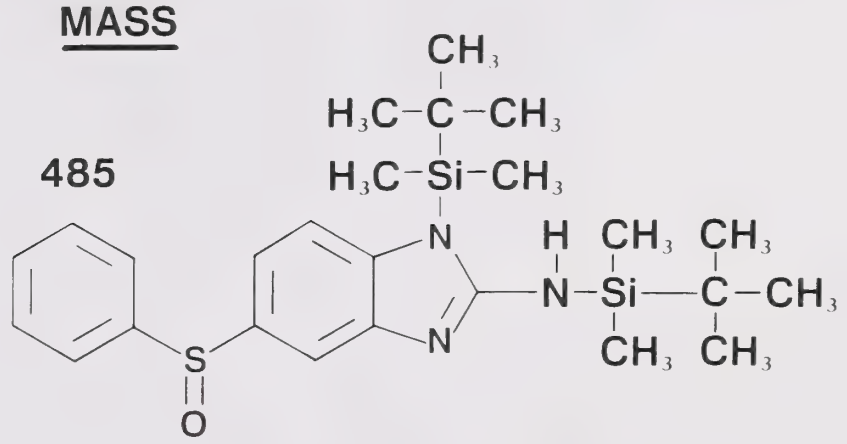
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

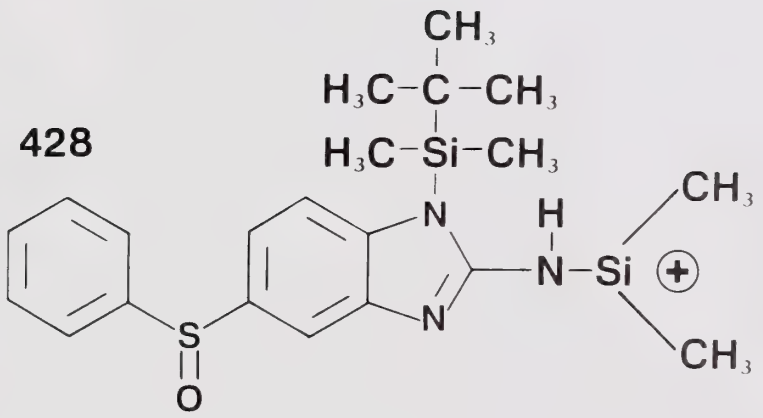
Postulated Structures of Oxfendazole Derivatives Used for MS Confirmation

MASS

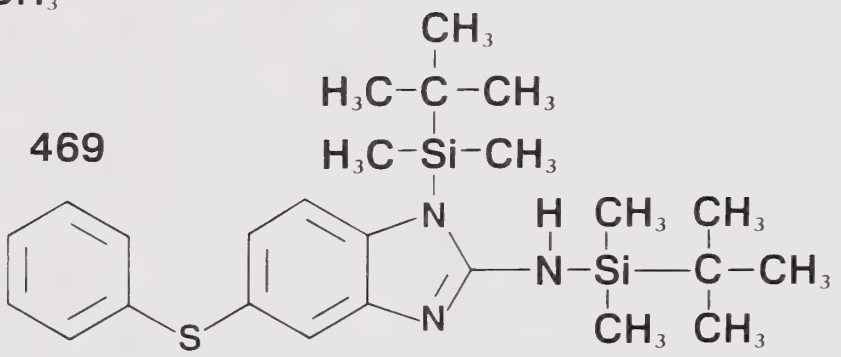
485



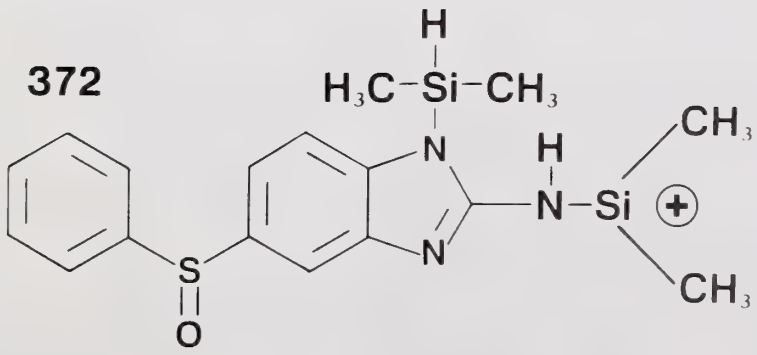
428



469



372



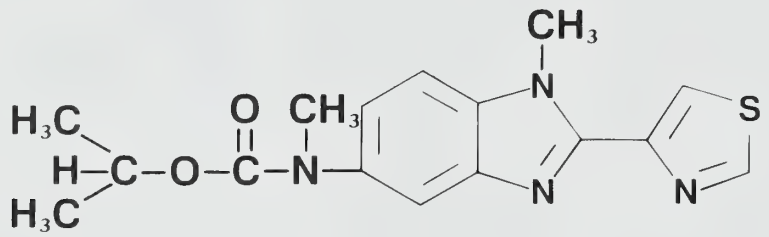
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

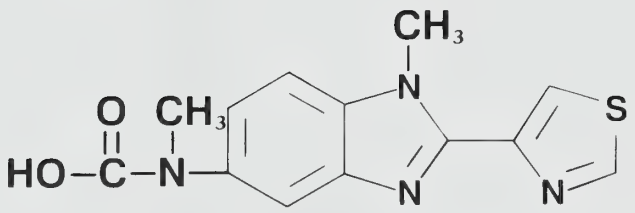
Postulated Structures of Cambendazole Derivatives Used for MS Confirmation

MASS

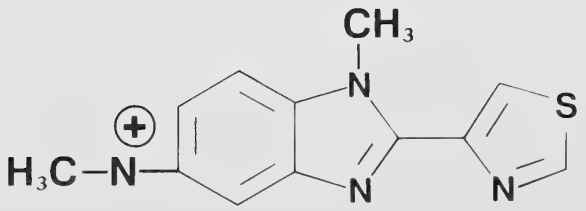
330



288



243



II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

See Determinative Method, section H, BNZ-24.

I. WORKSHEET

The worksheet on the following page, *Mass Spectrometry Data Form*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte:

Tissue:

	Ion Abundance m/z						*Ion Ratios*		
Blank									
Standard									
Fortified Sample									
Sample #									

Concentration:

Standard:

Fortified sample:

Sample:

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards.

a. No false standards at 0 ppb for all compounds.

b. No false negatives at the actionable concentrations.

2. Critical Control Points and Specifications

Intraset

a. Step II.E.2, heat at 110° C for 1 hour.

b. Step II.E.6, add 50 μL MTBSTFA and heat at 105° C for 2 hours.

3. Readiness To Perform

a. Run 1 blank for each tissue/species.

b. Run 1 sample at level of interest for each tissue/species.

c. Must be able to meet Criteria for Confirmation (II.F.3) when analyzing above samples.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst containing at least one of the benzimidazoles and using different species. At least one out of four of the positive samples is to be confirmed by mass spectrometry.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of Readiness To Perform (I.J.3) if cause was analyst-related.

5. Sample Acceptability and Stability

See Determinative Method, section J.5.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	a. Standards. b. Tissue blank. c. Tissue fortified at action levels.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): 50 ppb.

CHLORAMPHENICOL

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 3
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 9
G. Calculations 13
H. Hazard Analysis 14
I. Worksheet 16
J. Quality Assurance Plan 19

II. Confirmatory Method

A. Introduction 21
B. Equipment 21
C. Reagents 21
D. Standards 21
E. Derivatization 21
F. Analytical Confirmation Procedure 22
G. [Reserved]
H. Hazard Analysis 28
I. Worksheet 28
J. Quality Assurance Plan 31

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Chloramphenicol (CAP) has a broad spectrum of activity against gram-positive and gram-negative bacteria and rickettsia. It is widely used as an antimicrobial agent. It is banned in the U.S. for use in animals used as food.

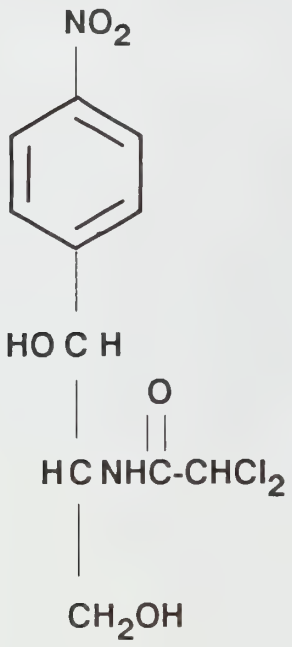
Meta-chloramphenicol is added to the sample as a recovery index. The sample is then incubated with β-glucuronidase to convert any chloramphenicol monoglucuronide to free chloramphenicol. CAP is extracted from muscle with ethyl acetate and the ethyl acetate is concentrated to about 1 mL. A 4 % sodium chloride solution is added and the remaining ethyl acetate is purged with nitrogen. The salt solution is applied to the top of a C_{18} SPE column, the cartridge is washed with methanol:water (20:80) and the CAP is eluted with methanol. The eluate is evaporated to dryness and silanized. CAP is estimated by GC/EC using a DB-1 capillary column. Confirmation is accomplished by GC/MS, using an OV-1 capillary column and negative ion chemical ionization.

2. Applicability

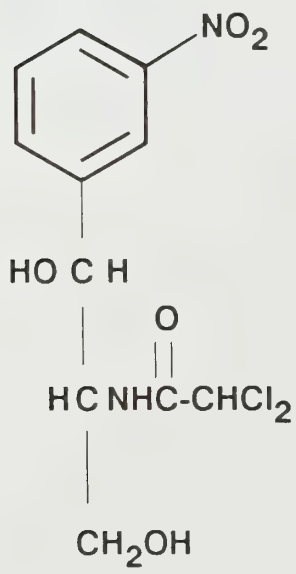
This method is applicable for veal calf muscle.

DETERMINATIVE METHOD

A. INTRODUCTION (Continued)



CHLORAMPHENICOL



METACHLORAMPHENICOL

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Vacuum manifold device, for aid in washing and elution of C_{18} cartridges. Optional, but allows for multiple C_{18} elutions. J. T. Baker or Analytichem.

b. N-Evap, Organomation Associates, or equivalent.

c. Pipettes, disposable, glass serological (10 mL) and Eppendorf (50-200 μL), or equivalent.

d. Test tube racks.

e. 50 mL Corex glass round-bottom centrifuge tubes with Teflon-lined screw caps, Corning #8422A, or equivalent.

f. Microliter syringes, 10 μL, for injection. Hamilton #701 and #1701, or equivalent.

g. Centrifuge, Damon IEC Division Model #PR-7000 with IEC #253 rotor, cup size IEC catalog #320, or equivalent.

h. Vortex mixer, Labline Supermixer Model #1290, or equivalent.

i. Homogenizer, Ultra Turrax, Tekmar Model SDT, with microshaft, or equivalent.

j. Incubator, low temperature, Precision Scientific Freas Model 825, or equivalent.

k. Heating module, Reacti-therm, Pierce Model #18780, or equivalent.

l. Conical 1 mL autosampler vials, available from Chemical Research Supplies, combo pack with polyethylene P8-6, or equivalent.

m. Culture tubes, 10 mL borosilicate, dimensions 13 $\times$ 10 mm, Corning #99445, or equivalent.

n. Pasteur pipettes, Kimble #72050, or equivalent.

o. Baker 10 SPE C_{18} (octadecyl) columns, 3 mL capacity, Catalog No. 7020-3, or similar C_{18} cartridge.

p. Borosilicate glass disposable centrifuge tube, 15 mL screwthread, Kimble #73785-15 and phenolic cap (PTFE faced, rubber liner), Kimble #73802-15415, or equivalent.

2. Instrumentation

a. Gas chromatograph, Hewlett-Packard 5880A with capillary inlet (splitless injection), fitted with electron capture Ni-63 detector, or equivalent.

b. Gas chromatographic column,DB-1, 30 meter length, 0.254 mm i.d., having a film thickness of 0.25 μm, J&W Scientific, or equivalent.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Methanol, Burdick and Jackson high-purity solvent product 230-4, or equivalent.

b. Ethyl acetate, Omni Solv product EXO241-1, or equivalent.

c. Hexane, UV, Burdick and Jackson high-purity solvent product 216, or equivalent.

d. Chloroform, Omni Solv product CX1054-1, or equivalent.

e. High-purity water, 18 megaohm/cm specific resistance.

f. Type IX β-glucuronidase, Sigma #G7396. Dilute with buffer (refer to item i below) to a concentration of 4,000 units/mL. Prepare fresh daily.

g. Acetonitrile, UV, Burdick and Jackson high-purity solvent product 015, or equivalent supplier

h. Cyclohexane, pesticide grade, Fisher C-553, or equivalent supplier.

i. Buffer solution containing 0.1M each of KH_2PO_4 and Na_2HPO_4 (ACS reagent grade) aqueous, pH 6.8 $\pm$ 0.1. pH is adjusted to 6.8 with the appropriate dry reagent.

j. Sylon HTP, Supelco #3-3-43, or equivalent.

Hexamethyldisilazane (HMDS)	3 parts
Chlorotrimethylsilane	1 part
Pyridine	9 parts

k. Sodium chloride, ACS grade. Prepare 4% aqueous solution with distilled water. Store at room temperature.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Drug standard.

Chloramphenicol, 99+% pure.
Sigma Chemical Co.
P.O. Box 14508
St. Louis, MO 63178

b. Internal standard.

Metachloramphenicol
USDA, FSIS, Midwestern Lab
Standards Repository
P.O. Box 5080
St. Louis, MO 63115
Attn: Carolyn Henry, CIC

2. Preparation of Standards

a. Prepare individual 500 μg/mL stock solutions of chloramphenicol and metachloramphenicol by transferring 50 mg standard to a 100 mL volumetric flask and diluting to volume with methanol.

b. Prepare individual intermediate solutions at 50 μg/mL by transferring 10 mL of stock into a 100 mL volumetric flask and diluting to volume with methanol.

c. Prepare individual working solutions at 100 ng/mL by transferring 200 μL of the intermediate solution into a 100 mL volumetric flask and diluting to volume with methanol.

3. Storage Conditions

All standards should be stored in amber glassware at −4° C.

4. Shelf Life Stability

Stock and intermediate standards: 6 months.
Working standards: One month.

This is applicable to both internal and chloramphenicol standards when stored under the above conditions.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 10 g previously ground muscle tissue into a 50 mL centrifuge tube.

b. To each sample add 100 μL metachloramphenicol internal standard (100 ng/mL in methanol, 1 ppb).

c. Prepare 1 blank muscle and 3 fortified blank muscle samples to be analyzed with each sample set. Add internal standard to the control tissues. Fortify the recovery samples at 0.5 ppb (50 μL of working standard), 1.0 ppb (100 μL of working standard), and 2.0 ppb (200 μL of working standard). Data generated from fortified samples will be used for calculations.

d. Add 15 mL phosphate buffer (pH 6.8 $\pm$ 0.1) and 200 μL β-glucuronidase (800 units) solution to all blanks, fortified controls, and sample tubes.

e. Blend in a tissuemizer for 30-60 sec at room temperature.

f. Incubate all tubes 90 min at 37° C. After incubation, samples may be left in refrigerator overnight.

g. Equilibrate tubes to room temperature.

h. Add 15 mL ethyl acetate to each tube.

i. Mix tubes on vortex mixer for 30 sec to extract chloramphenicol.

j. Centrifuge at 2,000 rpm (1000 G) for 2 min to separate phases.

k. Remove ethyl acetate (upper phase) with a disposable pipette and transfer to a clean 50 mL tube.

l. Repeat extraction of sample (steps h-k) and combine extracts.

m. Reduce ethyl acetate volume to 1 mL on an N-Evap under a gentle stream of nitrogen, using a sand bath temperature of approximately 60° C.

n. Add 4 mL aqueous 4% NaCl solution to all tubes and vortex for 5-10 sec.

o. Continue evaporation of ethyl acetate on N-Evap until ethyl acetate layer is absent, leaving a small oily residue.

p. Add 5 mL hexane to the 4 mL 4% aqueous NaCl layer. Vortex for 10 sec. Centrifuge at 2,000 rpm (1000 G) for 1 min. Remove top layer and discard.

q. Repeat step p.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

NOTE: Steps r through u should be performed immediately, one after the other. Do not allow the sorbent to dry.

r. Prepare a C_{18} column for each sample, blank, and fortified control by washing each C_{18} column sequentially with 5 mL methanol, 5mL chloroform, 5mL methanol, and 10 mL distilled water. Discard all washes.

s. Load the entire aqueous extract onto the C_{18} column using a disposable Pasteur pipette. Discard the eluate.

t. Rinse the sample tube by vortexing twice with 1 mL distilled water and adding the rinses onto the C_{18} column. Discard eluate.

u. Wash each C_{18} column with 1 mL water followed by 2 mL methanol:water (20:80). Allow the last wash to elute completely through the column. Discard washes.

v. Elute the chloramphenicol from the C_{18} column with acetonitrile, 2 × 1.5 mL, collecting the eluate in a clean 10 mL culture tube.

w. Evaporate the acetonitrile eluate to approximately 0.5 mL (*not to dryness!*) on an N-Evap using a sand bath temperature of 60° C and a gentle stream of nitrogen.

x. Transfer to 1 mL conical vial. Rinse the 10 mL tube by vortexing 5 sec with 0.5 mL acetonitrile and add to the 1 mL vial. Evaporate to dryness gently with nitrogen on Reacti-therm at 60° C.

CAUTION: Avoid moisture from this point forward.

y. To the dried residue, add 200 μL Sylon HTP.

z. Stopper and vortex 5 sec. React at 60-70° C in a sand bath for 15 min.

aa. Evaporate excess reagents on Reacti-therm at 60° C with gentle stream of nitrogen to approximately 10 μL.

CAUTION: Excessive drying time at this step may result in loss of analyte.

bb. Reconstitute residue in 100 μL cyclohexane/hexane (60:40). Vortex 5 sec.

cc. Inject suitable microliter volume of derivatized material into GLC for quantitative determination, or into GC/MS for confirmation.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary

Weigh 10 g ground muscle tissue.
Add internal standard.

10 g muscle tissue + 15 mL buffer + 800 units enzyme

Tissuemize.
Incubate 90 min @ 37° C.

Buffered sample + 15 mL EtOAc

Mix.
Centrifuge @ 2000 rpm.
Transfer EtOAc to a clean 50 mL tube.
Combine EtOAC extracts.
Evaporate to 1 mL @ 60° C.

2×

EtOAc (1 mL) + 4 mL 4% NaCl

Evaporate remaining EtOAc.

4 mL 4% NaCl + 5 mL hexane

Vortex.
Centrifuge @ 2000 rpm.
Remove top layer and discard.

2×

4 ml 4% NaCl

Apply to conditioned C_{18} SPE column.
Wash with 1 mL waxer.
Wash with 2 mL 20:80 MeOH:H_2O.
Discard washes.
Elute with 2 × 1.5 mL ACN.
Evaporate ACN to dryness *gently*.

Dried residue + 200 μL Sylon HTP

React 15 min @ 60%.
Evaporate excess reagent.
Reconstitue in 100 μL 60:40 cyclohexane/hexane.
Inject (GC/EC or GC/MS).

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

The following conditions are for the 5880A Hewlett-Packard GC as described in section B.2 and should be considered an example only. The analyst should optimize these parameters for the instrument being used.

a. Carrier gas:	Helium, linear velocity 29 cm/sec.
b. Make-up gas:	Argon/methane, 95/5, flow rate 50 mL/min.
c. Initial column temperature:	80° C, hold for 1 min.
d. Temperature programming:	Programmed at 30° C/min to 260° C; hold for 10 min or until the meta isomer and chloramphenicol have eluted. Then programmed at 30° C/min to 300° C; hold for 5 min to make sure all the sample has eluted.
e. Injector temperature:	280° C.
f. Detector temperature:	350° C.
g. Sensitivity setting:	2/8 attenuation.
h. Expected retention time:	Chloramphenicol 10 to 11 min; metachloramphenicol 9.5 to 10.5 min.
i. Expected response:	50% full-scale deflection for 0.20 ng chloramphenicol.

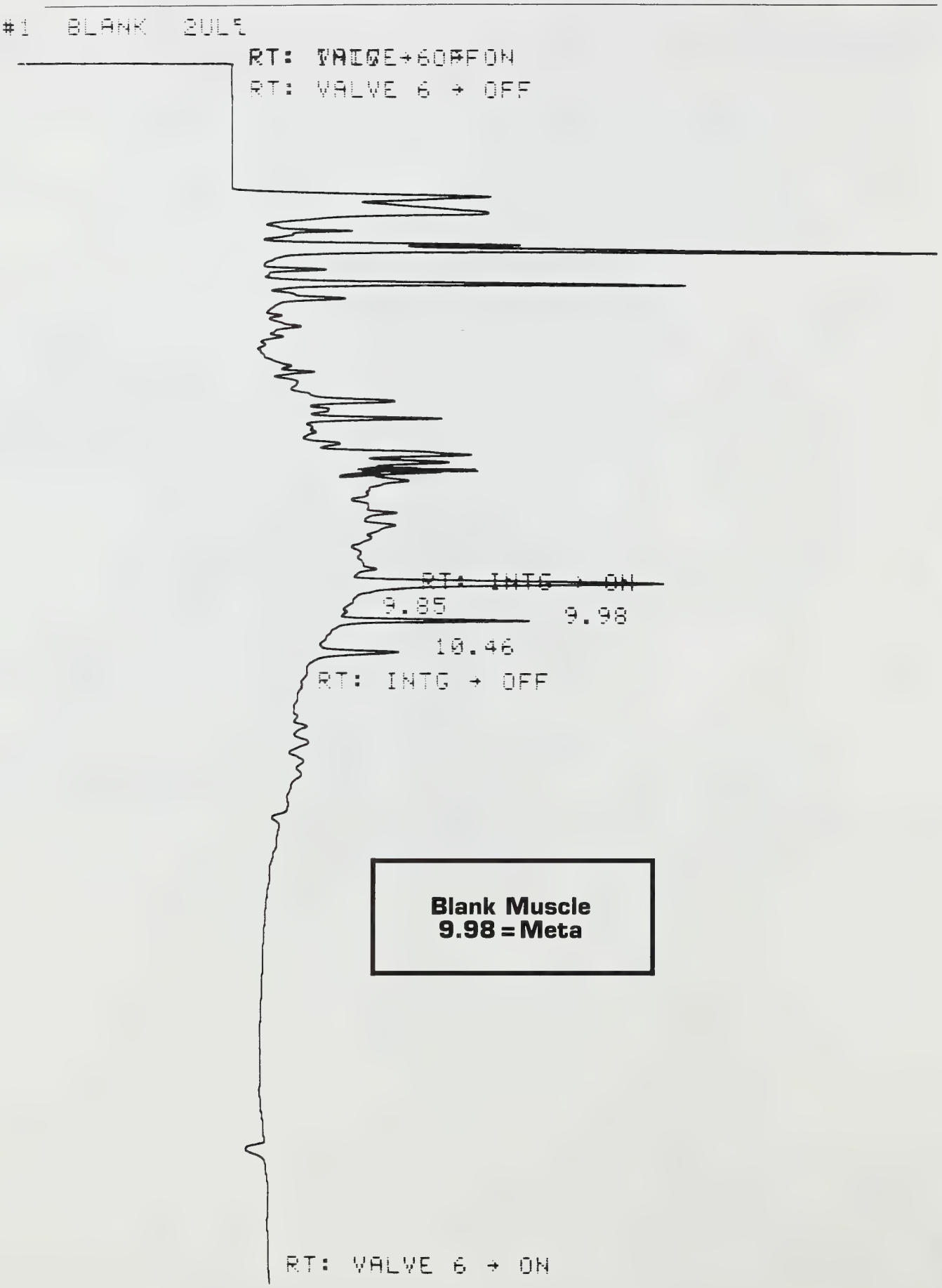
2. Interferences

No known interferences.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

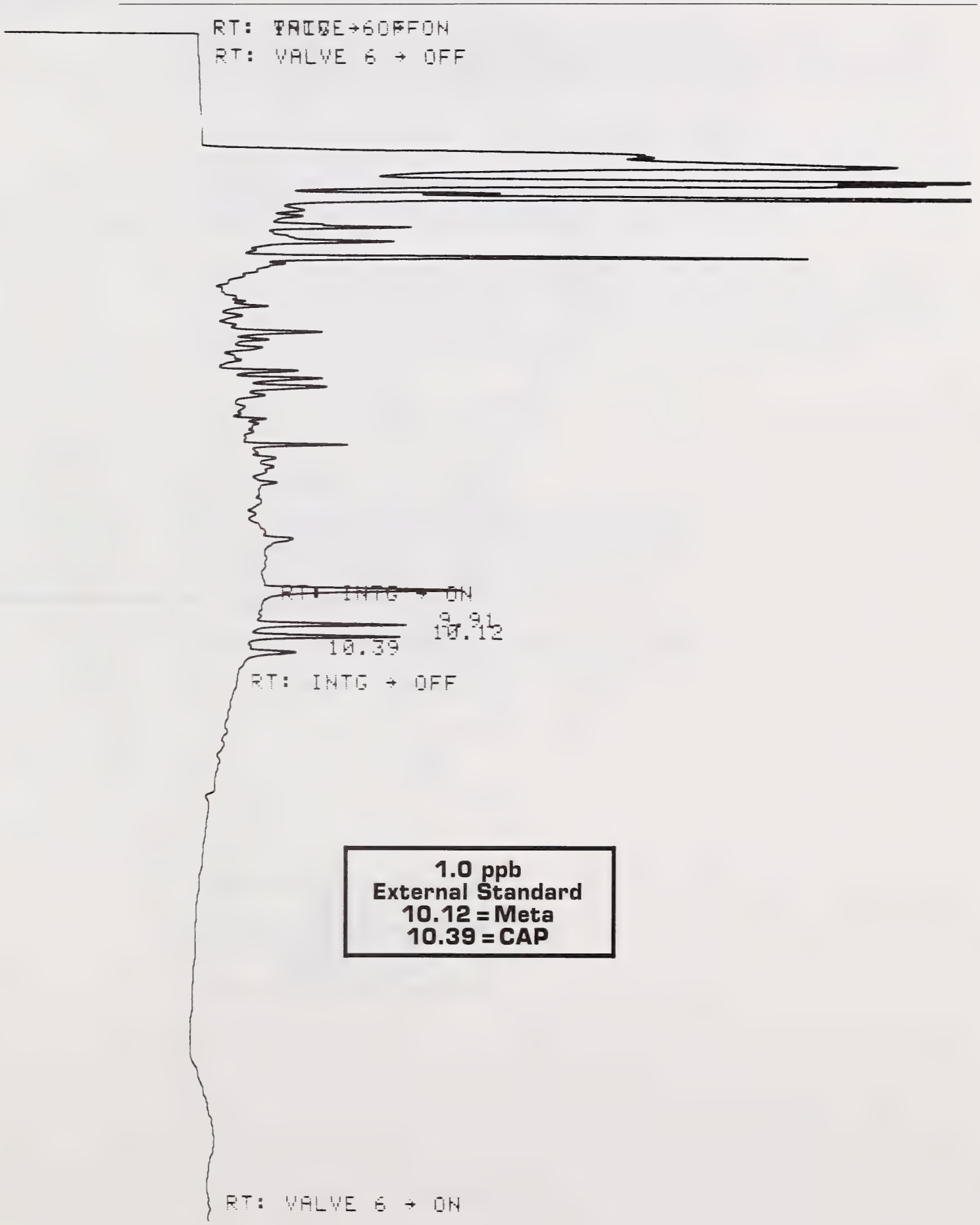
3. Sample Chromatograms



Blank Muscle
9.98 = Meta

I. DETERMINATIVE METHOD

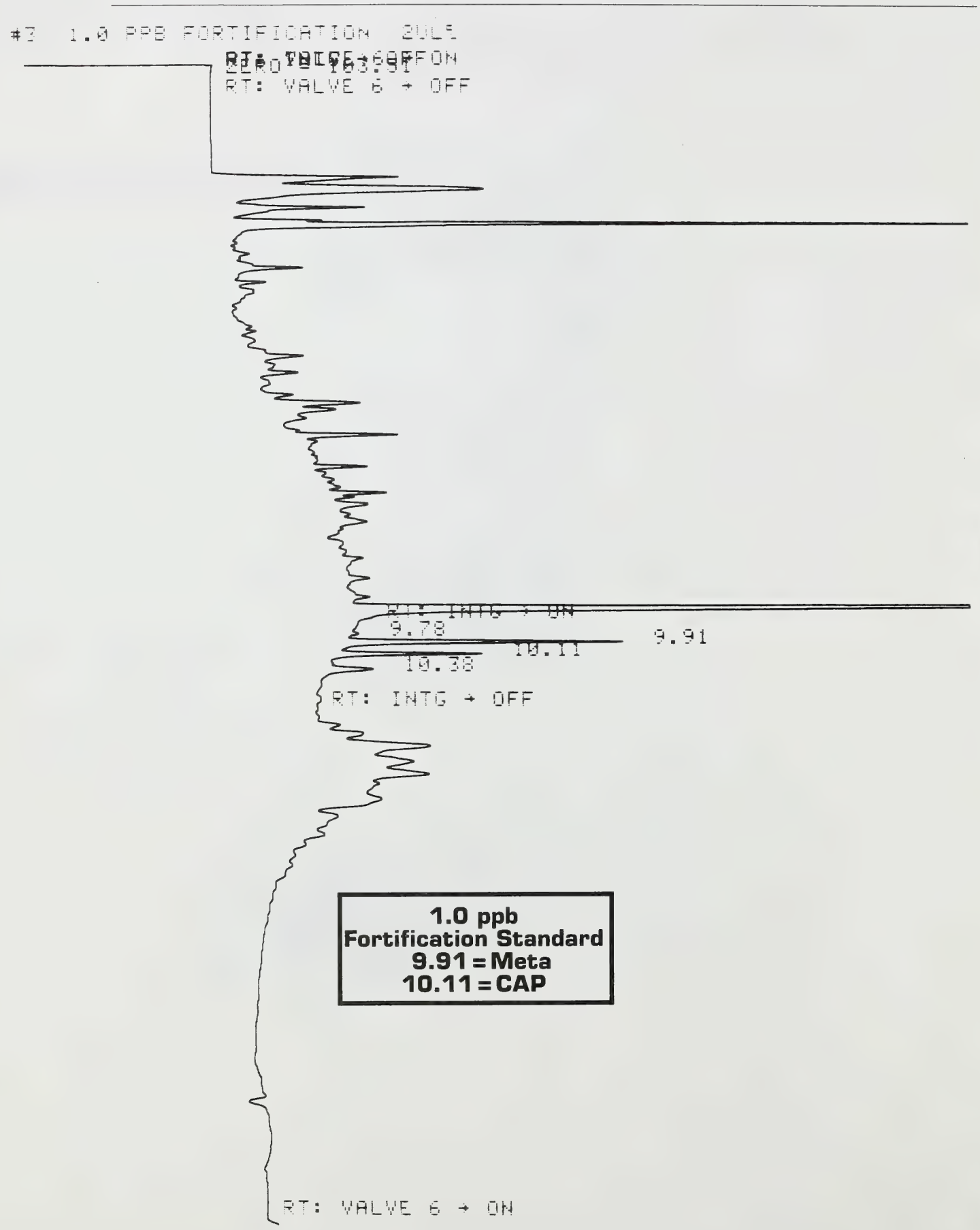
F. ANALYTICAL QUANTITATION (Continued)



1.0 ppb
External Standard
10.12 = Meta
10.39 = CAP

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



1.0 ppb
Fortification Standard
9.91 = Meta
10.11 = CAP

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Metachloramphenicol is used as an internal standard for calculating chloramphenicol concentration. Proceed in the following manner to calculate linear regression calibration curves and chloramphenicol concentration.

By an acceptable means, measure the peak height or peak area for each component in the 0.5 ppb, 1.0 ppb, and 2.0 ppb spiked samples that have been processed through the procedure. Calculate the peak height or peak area ratios for chloramphenicol by dividing its peak height or peak area by that of the metachloramphenicol peak height or peak area.

b. Using the ratios and associated ppb values, calculate a linear regression calibration curve by least squares computation as indicated in the Chemistry Quality Assurance Handbook Vol. II, Section 1.5.69. These calibration curves are then used to calculate values for additional samples from the sample set.

$y = mx + b$, where

m = slope

b = intercept

$$y = \frac{\text{chloramphenicol peak height or area}}{\text{metachloramphenicol peak height or area}}$$

x = chloramphenicol concentration in ppb

Standard curve correlation coefficient ≥ 0.9945

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Determination and Confirmation of Chloramphenicol in Veal Calf Muscle.

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat, and efficient fume hood.

3. Procedure Steps — CAUTION: Do not swallow, inhale, or absorb through skin any chemical, as complete toxicological properties for most chemicals are unknown.

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Enzyme: β-glucuronidase, type IXa	The toxicological properties have not been thoroughly investigated. May be harmful.	The analysis should be done under an efficient fume hood.
Solvents: Ethyl acetate, hexane, methanol, cyclohexane, chloroform.	Flammable, and the vapors are corrosive to the skin, eyes, and respiratory system. Chloroform is a possible carcinogen.	These solvents should be used only in an efficient fume hood, away from any electrical heating devices.
Analyte: Chloramphenicol Metachloramphenicol	Chloramphenicol has a wide range of possible adverse effects ranging from dermatitis, to embryo and fetal death, to death of adults. Chloramphenicol has been associated with inducing aplastic anemia. Those who survive aplastic anemia have a high incidence of developing acute leukemia. It is considered a carcinogen. The meta isomer is assumed to have similar toxicity.	Because the unique toxicity to humans has been well documented, the utmost care in handling chloramphenicol is recommended. See the material safety data sheet for information on how to handle any spills.
Derivatizing Reagents: 9:3:1 pyridine: hexamethyldisilazane: trimethylchorosilane	Flammable. This mixture can burn eyes, skin and may cause headache, nausea, dizziness, liver and kidney damage. Trimethylchlorosilane may be a possible carcinogen.	Use only in an efficient fume hood.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS (Continued)

4. Disposal Procedures	Organic solvents	See above	Store in the non-chlorinated solvent waste container until disposed of by the contractor or in-house specialist.
	Halogenated solvents	See above	Store in the chlorinated solvent waste container until disposed of by the contractor or in-house specialist.
	Chloramphenicol solutions	See above	Store separately in a well-marked container and hold for the contractor.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Chloramphenicol*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: CHLORAMPHENICOL

Analyst: _____ Matrix: _____

Date Started: _____ Date Completed: _____

Age of working standards:

Chloramphenicol: _____ Meta isomer: _____

Sample size: _____

Incubation time: At least 90 minutes

Sylon HTP volume: 200 μL ± 20 μL

Reaction temperature: 65 C ± 5 C

Final volume: 100 μL

Remarks:

Fortification Curve (ppb)	Retention Time-minutes meta CAP	Peak Height or Area Counts meta CAP	CAP:meta	Linear Regr
Control				b =
				m =
				r =
				s =

Sample Identification	Retention Time-minutes meta CAP	Peak Height or Area Counts meta CAP	CAP:meta	Amt Found (ppb)

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Compound	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability CV %*
Chloramphenicol	0.5-2.0	50-60	<20 at 1.0 ppb

2. Critical Control Points and Specifications

a. Intraset.

Record	*Acceptable Control*
β-glucuronidase solution	Prepare fresh daily.
Incubation time	Minimum incubation time is 90 min.
Incubation temperature	37° ± 2° C.
Sample size	10.0 ± 0.1 g.
Working standard solution volume	Depends on fortification level.
Sylon HTP volume	200 μL ± 20 μL.
Reaction time	15 min ± 1 min.
Reaction temperature	65° ± 5° C.
Evaporation of excess Sylon HTP	See section E.1.x.

b. Interset.

Age of working standard solution	One month.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each, on 3 separate days:

(a) Blank (0 ng/μL).

(b) 0.5 ppb (0.05 ng/μL).

(c) 1.0 ppb (0.10 ng/μL).

(d) 2.0 ppb (0.20 ng/μL).

NOTE: ppb calculated based on 10 g sample.

ii. Phase II: Fortified samples—4 levels, 6 replicates each. Same as above—over a minimum of three days, 24 total samples.

Submit data from standards to Chemistry Division for information.

NOTE: Phase I and II may be performed concurrently.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, PREB.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. Frequency

i. At least one check sample biweekly per analyst . At least one out of four of the positive samples is to be confirmed by mass spectrometry.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

(d) Fortification curve correlation data.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptablility and Stability

a. Sample Storage: Samples are stable for six months if stored at −80° C.

b. Condition upon receipt: Cold.

6. Sample Set

a. A tissue blank.

b. Fortified blanks at 0.5, 1.0, and 2.0 ppb.

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 0.5 ppb.

b. Lowest reliable quantitation (LRQ): 1.0 ppb.

II. CONFIRMATORY METHOD

A. INTRODUCTION

Refer to Determinative Method, section A.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.1

2. Instrumentation

GC/MS: Extranuclear Model 275, quadrupole, with capillary inlet, splitless injection fitted with an OV-1, 25 m × 0.20 mm id, fused silica crosslinked methyl silicone column, film thickness 0.33 μm (Hewlett Packard #19091A-102 or equivalent), capable of negative ion chemical ionization.

C. REAGENTS

a. Perfluorotributylamine (PFTBA)

b. Refer to Determinative Method, section C.

D. STANDARDS

a. See Determinative Method, section D.

b. The same sample set that is used in the Determinative Method will be used by the mass spectrometer operator for confirmation. Only the 0.5 ppb standard is usually analyzed.

E. DERIVATIZATION

Sample Preparation

A suitable microliter volume of derivatized material obtained in the determinative procedure (see Determinative Method, section E) is injected into the GC/MS for confirmation.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions and parameters.

NOTE: The following conditions are for the extranuclear instrument described in section B.2 and are given as an example only. The analyst should optimize the parameters for the instrument being used.

Column: OV-1 Fused Silica Capillary, 25 m.

Injector: 230° C.

Temperature programming: 150° C with no initial hold, programmed to 300° C at 20° C/min. Final hold 10 min.

Transfer line temperature: 300° C.

Helium flow rate: 29 cm/sec, splitless injection.

b. Mass spectrometer conditions.

Detection: Enhanced negative ion chemical ionization (NICI).

Ionization gas: Isobutane or methane.

Source pressure: 1.0×10^{-5} T.

Source temperature: 260° C.

Mode of operation: SIM-NICI.

Dwell time: 10 millisec.

Calibration standard: PFTBA; maximize on ion 452.

Scan width: 0.2 amu.

Expected signal/noise of lowest intensity ion: 10/1.

Filament voltage: 300 eV.

Filament current: 1000 μA.

2. Required Samples for GC/MS Analysis

Set up instrument as described in section 1 above to monitor chloramphenicol and the internal standard metachloramphenicol. Inject 2 to 5 μL of the external standard and analyze results to verify that the system is functioning properly. If not, make any necessary adjustments in operating parameters or standard concentration, then reinject to verify performance. Inject 2 to 5 μL each of the confirmation sample, the recovery, and the tissue blank. Confirmatory analysis is required in all samples found positive by the determinative method.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

a. Retention time specification. Compare the retention time of the sample with the retention time of the fortified tissue. The times should match ± 5.0%. The following is an example only as other retention times are acceptable.

Compound	*Expected Retention Time (min)*
Metachloramphenicol	8.2 ± 0.5
Chloramphenicol	9.0 ± 0.5

b. Ions 468, 466, 322, and 304 must be present. In samples with high levels of chloramphenicol, ions m/z 358 and 360 can also be monitored.

c. Compute at least the following three ratios for the standard, fortification, and the standard, 466/468, 322/466, and 304/466. For successful confirmation between levels 0.25 ppb and 1.0 ppb, the 466/468 and either the 322/466 or the 304/466 ion for the sample must agree within ± 20% of the ratio for fortified tissue. In samples with high levels of chloramphenicol, also compute ratios 358/466 and 358/360.

The ion ratios of the fortified standard must match the ion ratios of the sample by ± 20%. The following are expected ion ratios for the conditions cited.

466/468 = 1.43

322/466 = 0.49

304/466 = 0.57

NOTE: The ion ratios are dependent upon the source temperature.

Refer to the following page for postulated structured of confirming ions.

4. Interferences

No known interferences.

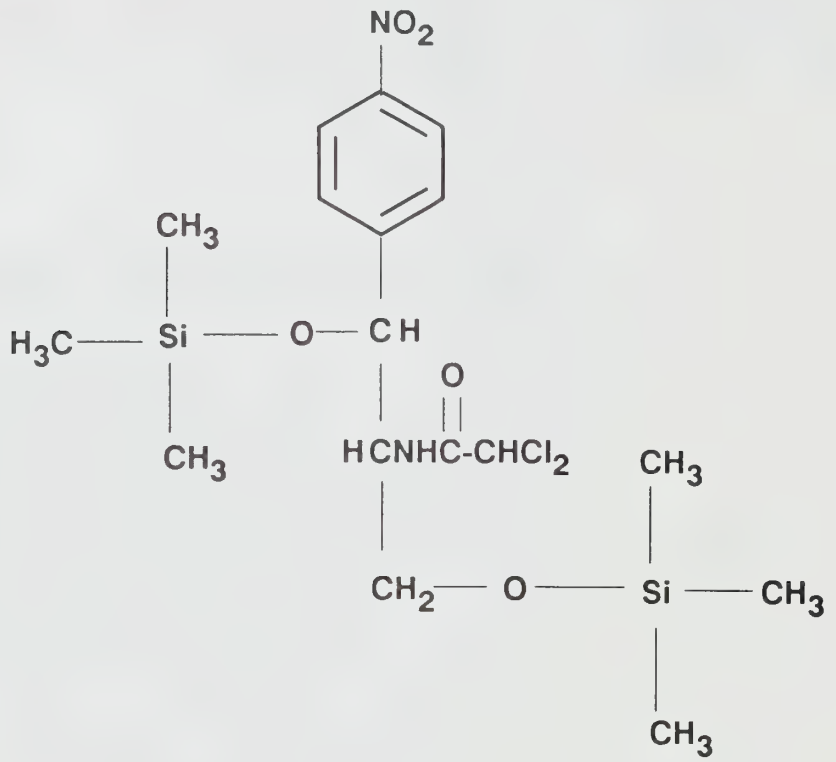
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Postulated structures of some confirming ions.

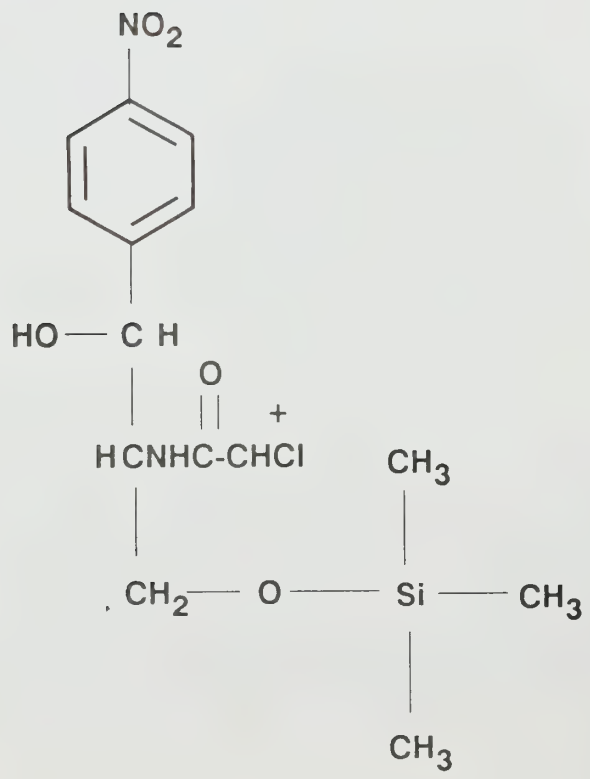
MASS

468 AND 466
Due to Cl isotopes



MASS

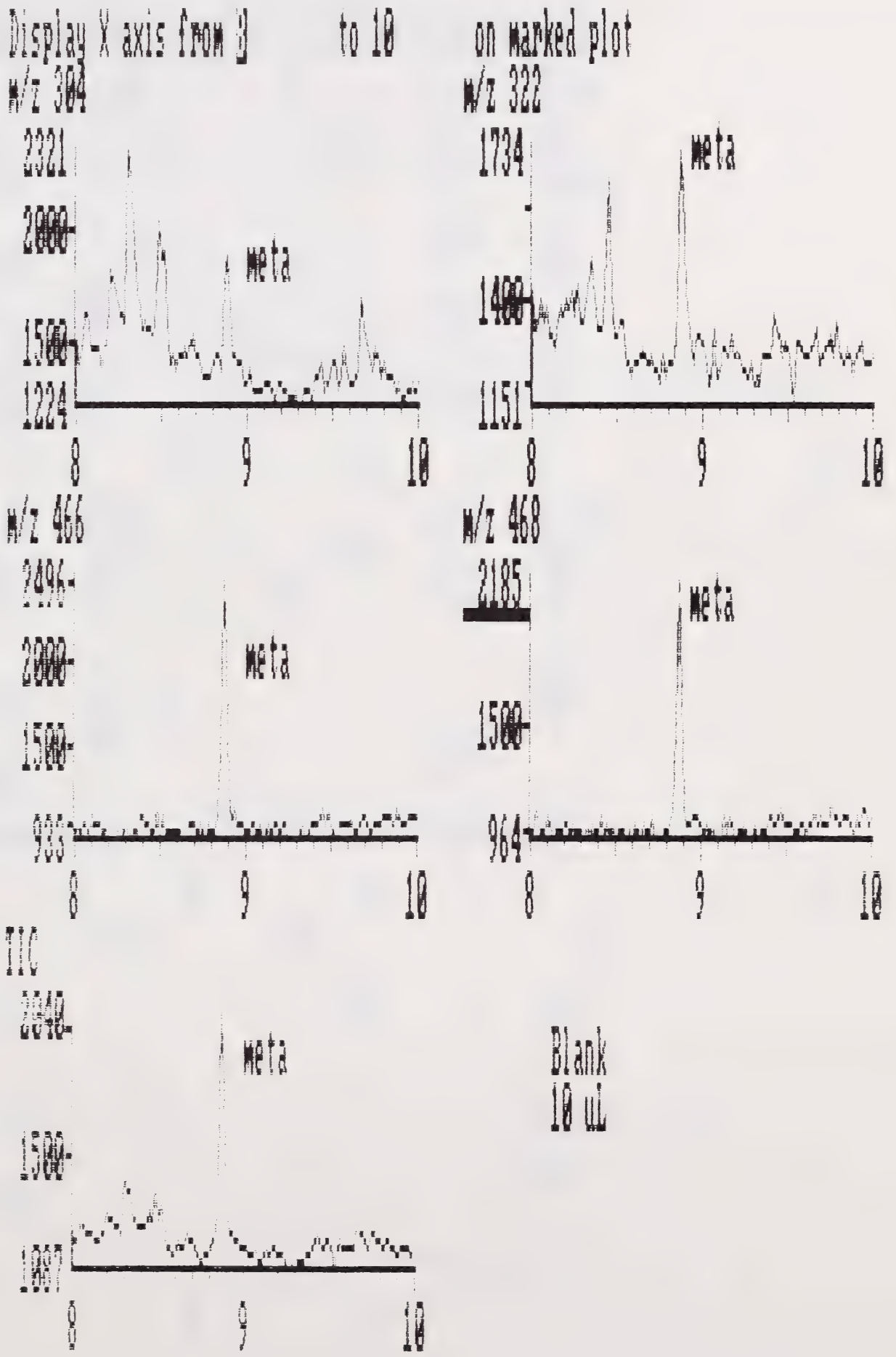
358



II. CONFIRMATORY METHOD

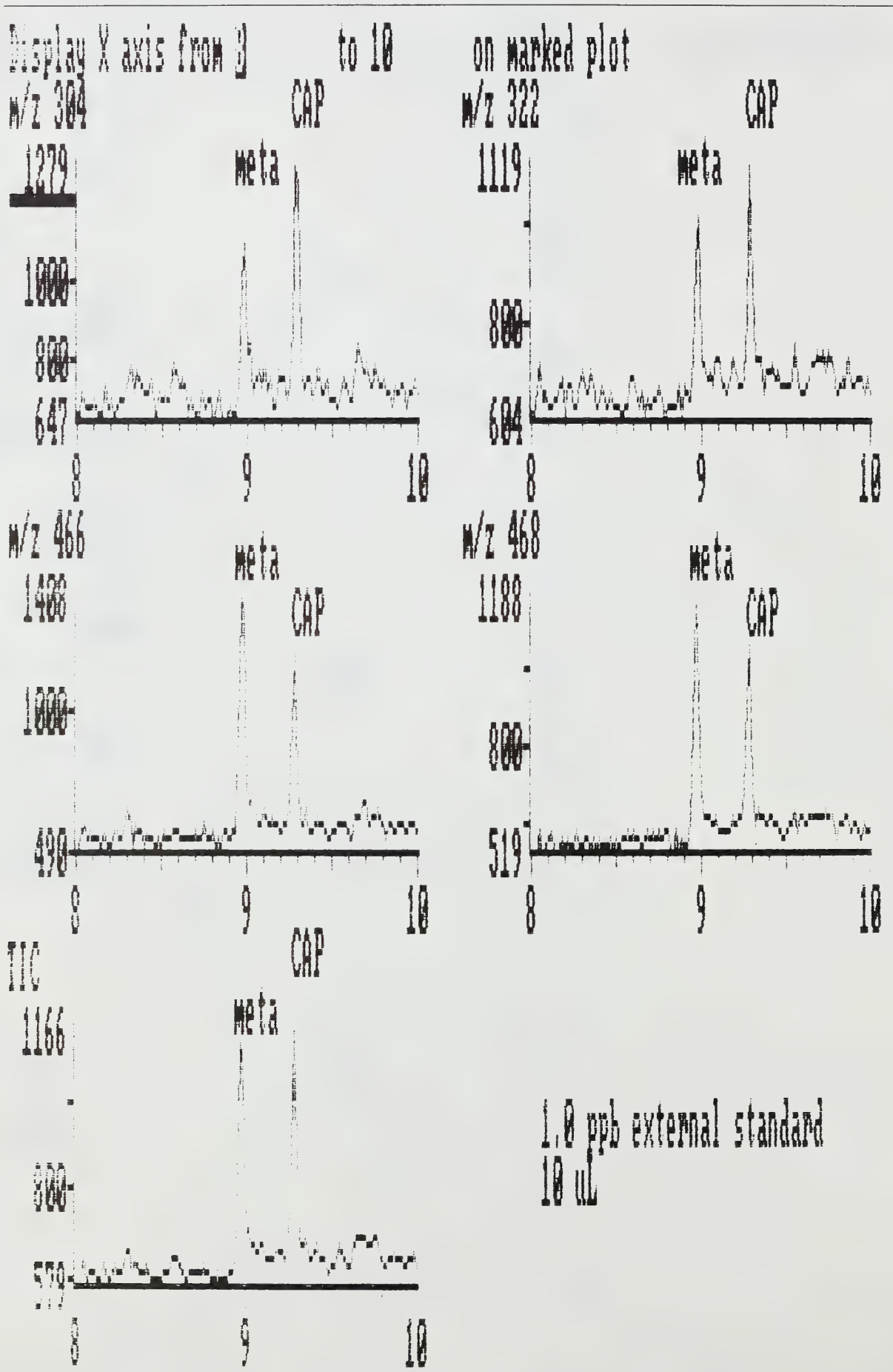
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

5. Sample Chromatograms



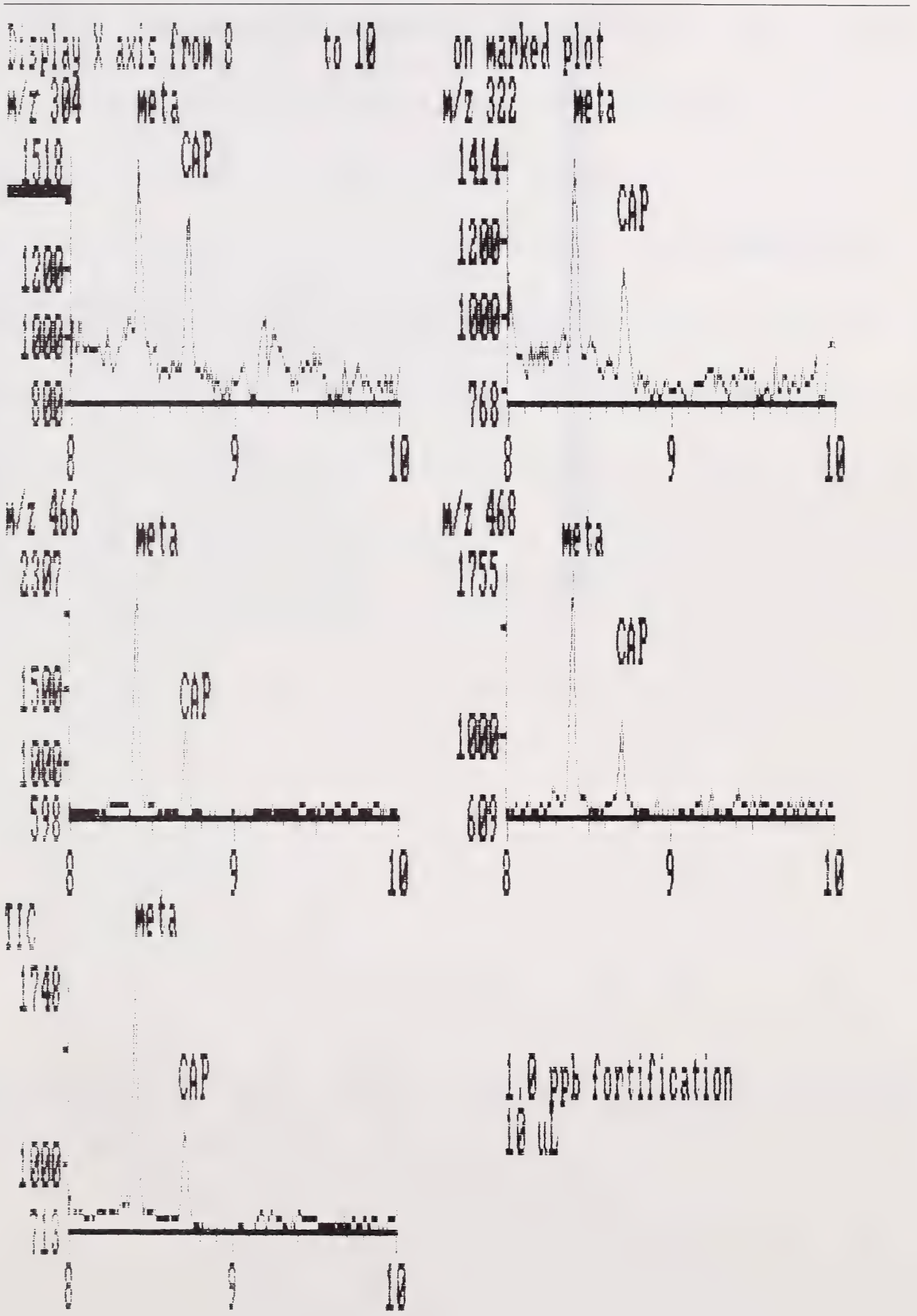
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

Refer to Determinative Method, section H.

I. WORKSHEET

The worksheet on the facing page, *Mass Spectrometry Data Form*, can be removed from this book can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte: _____ Tissue: _____

	R.T. (min) Base Peak	Ion Abundance (m/z)				Ion Ratios		
	_____	_____	_____	_____	_____	_____	_____	_____
Blank								
Standard								
Fortified Tissue								
Sample #								

Concentration:

Standard: _____

Fortified tissue: _____

Sample: _____

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb.

b. No false negatives at 1.0 ppb.

2. Critical Control Points and Specifications

See Determinative Method, section J.2.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standards, show that the instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives.

Submit data on standards and samples to Chemistry Division for information.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

i. No false positives or negatives.

ii. Must monitor at least 4 ions.

iii. Retention time of analyte peak must match the standard or fortified standard within 5%.

iv. Ratio of 466/468 ion pair, in addition to one other ion, must match those of the 1.0 ppb fortified standard by ± 20%.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

b. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for :

i. All replicate findings.

ii. Control chart on differences between replicates.

iii. All percent recoveries.

iv. Fortification curve correlation data.

c. Acceptability criteria.

If values are unacceptable:

i. Stop all offical analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Sample storage: Samples are stable for six months if stored at −80° C.

b. Condition upon receipt: Cold.

6. Set Construction

a. Standard.

b. Tissue Blank.

c. Tissue fortified at action levels.

d. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): Not applicable.

b. Lowest reliable confirmation (LRC): 0.5 ppb.

CHLORAMPHENICOL/URINE

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 8
G. Calculations 11
H. Hazard Analysis 12
I. Worksheet 14
J. Quality Assurance Plan 17

II. Confirmatory Method

A. Introduction 19
B. Equipment 19
C. Reagents 19
D. Standards 19
E. Derivatization 19
F. Analytical Confirmation Procedure 20
G. [Reserved]
H. Hazard Analysis 25
I. Worksheet 25
J. Quality Assurance Plan 29

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Chloramphenicol (CAP) has a broad spectrum of activity against gram-positive and gram-negative bacteria and rickettsia. It is widely used as an antimicrobial agent. It is banned in the U.S. for use in animals used as food.

Meta-chloramphenicol is added to the sample as a recovery index. The sample is then incubated with β-glucuronidase to convert any chloramphenicol monoglucuronide to free chloramphenicol. CAP is extracted from muscle with ethyl acetate and the ethyl acetate is concentrated to about 1mL. A 4 % sodium chloride solution is added and the remaining ethyl acetate is purged with nitrogen. The salt solution is applied to the top of a C_{18} SPE column, the cartridge is washed with 20:80 methanol:water and the CAP is eluted with 50:50 methanol:water. The eluate is diluted with water and applied to a second C_{18} column and the CAP is eluted with methanol. The eluate is evaporated to dryness, and silanized. CAP is estimated by GC/EC using a DB-1 capillary column. Confirmation is accomplished by GC/MS, using an OV-1 capillary column and negative ion chemical ionization.

Refer to CAM method, section A.1, for structure.

2. Applicability

This method is applicable for veal calf urine.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Vacuum manifold device, for aid in washing and elution of C_{18} cartridges. Optional, but allows for multiple C_{18} elutions. J. T. Baker, Analytichem, or equivalent.

b. N-Evap, Organomation Associates, or equivalent.

c. Pipettes, disposable, glass serological (10 mL) and Eppendorf (50-200 μL), or equivalent.

d. Test tube racks.

e. 50 mL Pyrex glass round-bottom centrifuge tubes with #13 ground-glass stoppers, Pyrex #8424, or equivalent.

f. Microliter syringes, 10 μL, for injection. Hamilton #701 and #1701, or equivalent.

g. Centrifuge, Damon IEC Division Model #PR-7000 with IEC #253 rotor, cup size IEC catalog #320, or equivalent.

h. Votex mixer, Labline Supermixer Model #1290, or equivalent.

i. Homogenizer, Ultra Turrax, Tekmar Model SDT, with microshaft, or equivalent.

j. Incubator, low temperature, Precision Scientific Freas Model 825, or equivalent.

k. Heating module, Reacti-therm, Pierce Model #18780, or equivalent.

l. Conical 1 mL autosampler vials, available from Chemical Research Supplies, combo pack with polyethylene P8-6, or equivalent.

m. Culture tubes, 10mL borosilicate, 13 × 100 mm, Corning #99445, or equivalent.

n. Pasteur pipettes, Kimble #72050, or equivalent.

o. Baker 10 SPE C_{18} (octadecyl) columns, 3 mL capacity, Catalog No. 7020-3, or similar C_{18} cartridge.

p. Borosilicate glass disposable centrifuge tube, 15 mL screwthread, Kimble #73785-15 and phenolic cap (PTFE faced, rubber liner), Kimble #73802-15415, or equivalent.

q. Microfilter, 0.45 μm, Gelman #4217 or equivalent.

2. Instrumentation

Gas chromatograph, Hewlett-Packard 5880A with capillary inlet, splitless injection, fitted with an electron capture Ni^{63} detector and with a DB-1 30 m × 0.25 mm id fused silica cross-linked methyl silicone column, film thickness 0.25 μm (J & W Scientific, #122-1032 or equivalent).

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Methanol, Burdick and Jackson high-purity solvent product 230-4, or equivalent supplier.

b. Ethyl acetate, Omni Solv product EXO241-1, or equivalent supplier.

c. Chloroform, Omni Solv product CX1054-1, or equivalent supplier.

d. High-purity water, 18 megaohm/cm specific resistance.

e. Type IXa β-glucuronidase, Sigma #G7396. Dilute with buffer (see item g below) to a concentration of 4,000 units/mL. Prepare fresh daily.

f. Cyclohexane, pesticide grade, Fisher C-553, or equivalent supplier.

g. Buffer solution containing 0.1M each of KH_2PO_4 and Na_2HPO_4 (ACS reagent grade) aqueous, pH 6.8 $\pm$ 0.1. pH is adjusted to 6.8 with the appropriate dry reagent.

h. Sylon, HTP, Supelco #3-3-43, or equivalent.

Hexamethyldisilazane (HMDS)	3 parts
Chlorotrimethylsilane	1 part
Pyridine	9 parts

i. Sodium chloride, ACS grade. Prepare 4% aqueous solution with distilled water. Store at room temperature.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Drug standard.

Chloramphenicol, 99+ percent pure.
Sigma Chemical Co.
P.O. Box 14508
St. Louis, MO 63178

b. Internal standard.

Metachloramphenicol
USDA, FSIS, Midwestern Lab
Standards Repository
P.O. Box 5080
St. Louis, MO 63115
Attn: Carolyn Henry, CIC

2. Preparation of Standards

a. Prepare individual 500 μg/mL stock solutions of chloramphenicol and metachloramphenicol by transferring 50 mg standard to a 100 mL volumetric flask and diluting to volume with methanol.

b. Prepare individual intermediate solutions at 50 μg/mL by transferring 10 mL of stock into a 100 mL volumetric flask and diluting to volume with methanol.

c. Prepare individual working solutions at 100 ng/mL by transferring 200 μL of the intermediate solution into a 100 mL volumetric flask and diluting to volume with methanol.

3. Storage Conditions

All standards should be stored in amber glassware at −4° C.

4. Shelf Life Stability

Both standard and internal standard working standards are stable for one month when stored under the above conditions. The stock and intermediate standards for both the standard and internal standard are stable for six months when stored under the above conditions.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Filter calf urine through a 0.45 μm pore filter. Pipet 5 mL of filtered urine into a 50 mL glass centrifuge tube.

b. To each sample add 250 μL metachloramphenicol internal standard (100 ng/mL in methanol, 1 ppb).

c. Prepare 1 blank urine and 3 fortified blank urine samples to be analyzed with each sample set. Add internal standard to the control tissues. Fortify the recovery samples at 2.5 ppb (125 μL of working standard), 5.0 ppb (250 μL of working standard), and 10.0 ppb (500 μL of working standard). Data generated from fortified samples will be used for calculations.

d. Add 1 mL phosphate buffer (pH 6.8 $\pm$ 0.1) and 200 μL β-glucuronidase (800 units) solution to all blanks, fortified controls, and sample tubes.

e. Mix on a vortex mixer for 5-10 sec at room temperature.

f. Incubate all tubes 90 min at 37° C. After incubation, samples may be left in refrigerator overnight.

g. Equilibrate tubes to room temperature.

h. Add 10 mL ethyl acetate to each tube.

i. Mix tubes on vortex mixer for 30 sec to extract chloramphenicol.

j. Centrifuge at 2,000 rpm (1000 G) for 2 min to separate phases.

k. Remove ethyl acetate (upper phase) with a disposable pipette and transfer to a clean 50 mL tube.

l. Repeat extraction of sample (steps h-k) and combine extracts.

m. Reduce ethyl acetate volume to 1 mL on an N-Evap under a gentle stream of nitrogen, using a sand bath temperature of approximately 60° C.

n. Add 4 mL aqueous 4% NaCl solution to all tubes and vortex 5-10 sec.

o. Continue evaporation of ethyl acetate on N-Evap until ethyl acetate layer is visually absent.

NOTE: Steps p through t should be performed immediately one after the other. Do not allow the sorbent to dry.

p. Prepare a C_{18} column for each sample, blank, and fortified control by washing each C_{18} column sequentially with 5 mL methanol, 5 mL chloroform, 5 mL methanol, and 10 mL distilled water. Discard all washes.

q. Load the entire aqueous extract onto the C_{18} column using a disposable Pasteur pipette. Discard the eluate.

r. Rinse the sample tube by vortexing with 2 $\times$ 1 mL distilled water and adding the rinses onto the C_{18} column. Discard eluate.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

s. Wash each C_{18} column with 1 mL water followed by 2 mL 20:80 methanol:water. Allow the last wash to elute completely through the column. Discard washes.

t. Elute the chloramphenicol from the C_{18} column with 3 mL 50:50 methanol:water, collecting the eluate in a clean 10 mL culture tube.

u. Transfer the eluate to a 15 mL disposable centrifuge tube. Rinse the 10 mL tube twice, vortexing for 5 sec, with 1 mL of distilled water.

v. Add 8 mL distilled water to each 15 mL centrifuge tube.

w. Prepare a second C_{18} column as in step p.

Note: Steps w-y should be performed immediately one after the other. Do not allow sorbent to dry.

x. Load the solution from step v onto the second C_{18} column. Rinse the sample tube by vortexing with 2 × 1 mL distilled water rinses and add the rinses to the column. Allow solution to elute completely through the column. Discard eluate.

y. Elute the chloramphenicol from the C_{18} column with 5 mL methanol, collecting the eluate in a clean 10 mL culture tube.

z. Evaporate the methanol eluate to 0.5 mL (*not to dryness*) on an N-Evap, using a sand bath temperature of 60° C and a gentle stream of nitrogen.

aa. Transfer to 1 mL conical vial. Rinse the 10 mL tube by vortexing 5 sec with 0.5 mL methanol and add to the 1 mL vial. Evaporate to dryness gently with nitrogen on Reacti-therm at 60° C.

CAUTION: Avoid moisture from this point forward.

bb. To the dried residue, add 200 μL Sylon HTP.

cc. Stopper and vortex 5 sec. React at 60-70° C in a sand bath for 15 min.

dd. Evaporate excess reagents on Reacti-therm at 60° C with gentle stream of nitrogen to approximately 10 μL.

CAUTION: Excessive drying time at this step may result in loss of analyte.

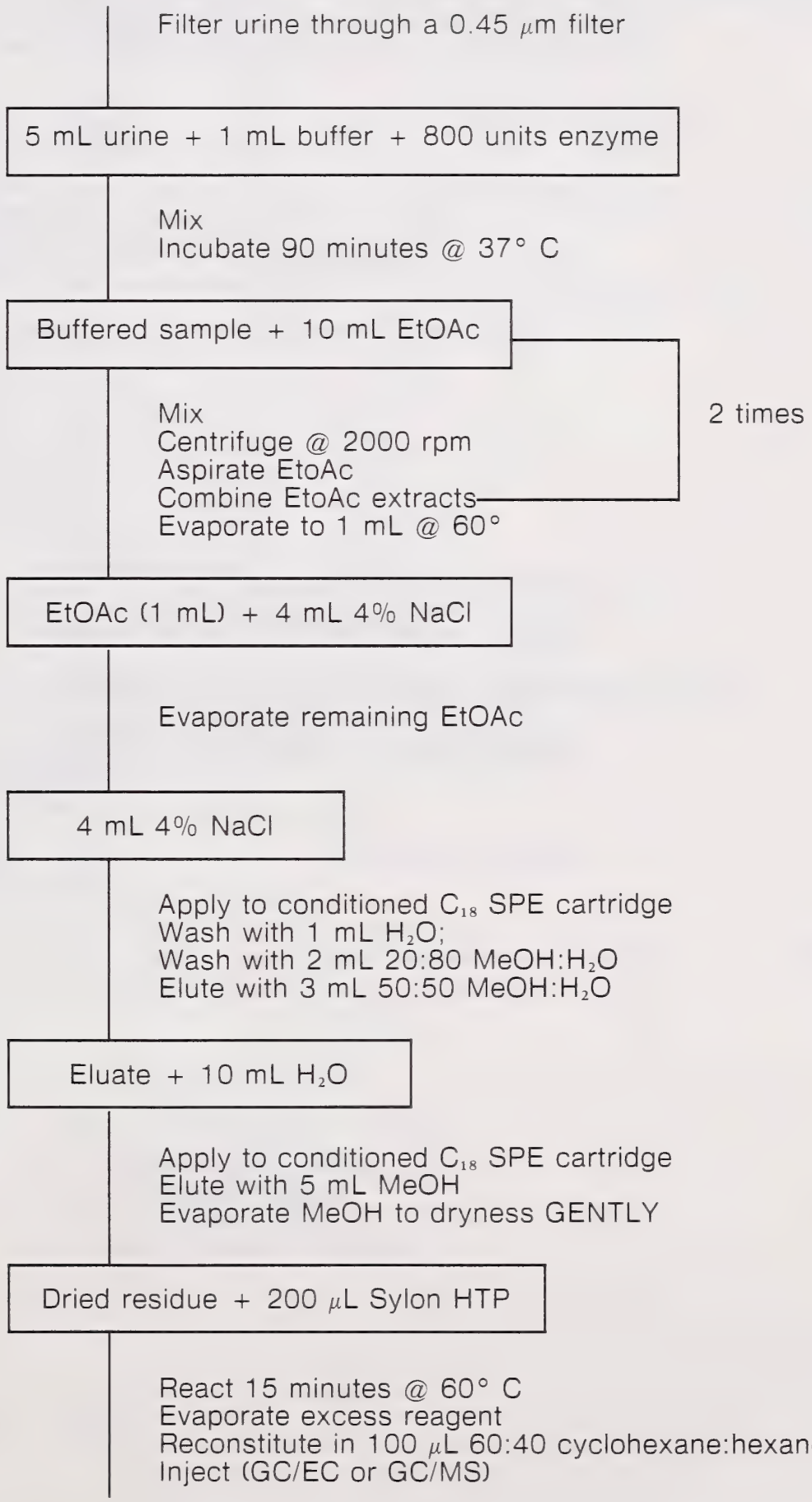
ee. Reconstitute residue in 100 μL 60:40 cyclohexane:hexane; vortex 5 sec.

ff. Inject suitable microliter volume of derivatized material into GLC for quantitative determination, or into GC/MS for confirmation.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

These conditions are for the 5880A Hewlett-Packard gas chromatograph described in section B.2 and are given as an example only. The analyst should optimize these parameters for the instrument being used.

a. Carrier gas:	Helium, linear velocity 29 cm/sec.
b. Make-up gas:	Argon/methane, 95/5, flow rate 50 mL/min.
c. Initial column temperature:	80° C, hold for 1 min.
d. Temperature programming:	Programmed at 30° C/min to 260° C; hold for 10 min or until the meta isomer and chloramphenicol have eluted. Then programmed at 30° C/min to 300° C; hold for 5 min to make sure all the sample has eluted.
e. Injector temperature:	280° C.
f. Detector temperature:	350° C.
g. Sensitivity setting:	218 attenuation.
h. Expected retention time:	Chloramphenicol 10 to 11 min; metachloramphenicol 9.5 to 10.5 min.
i. Expected response:	50% full-scale deflection for 0.20 ng chloramphenicol.

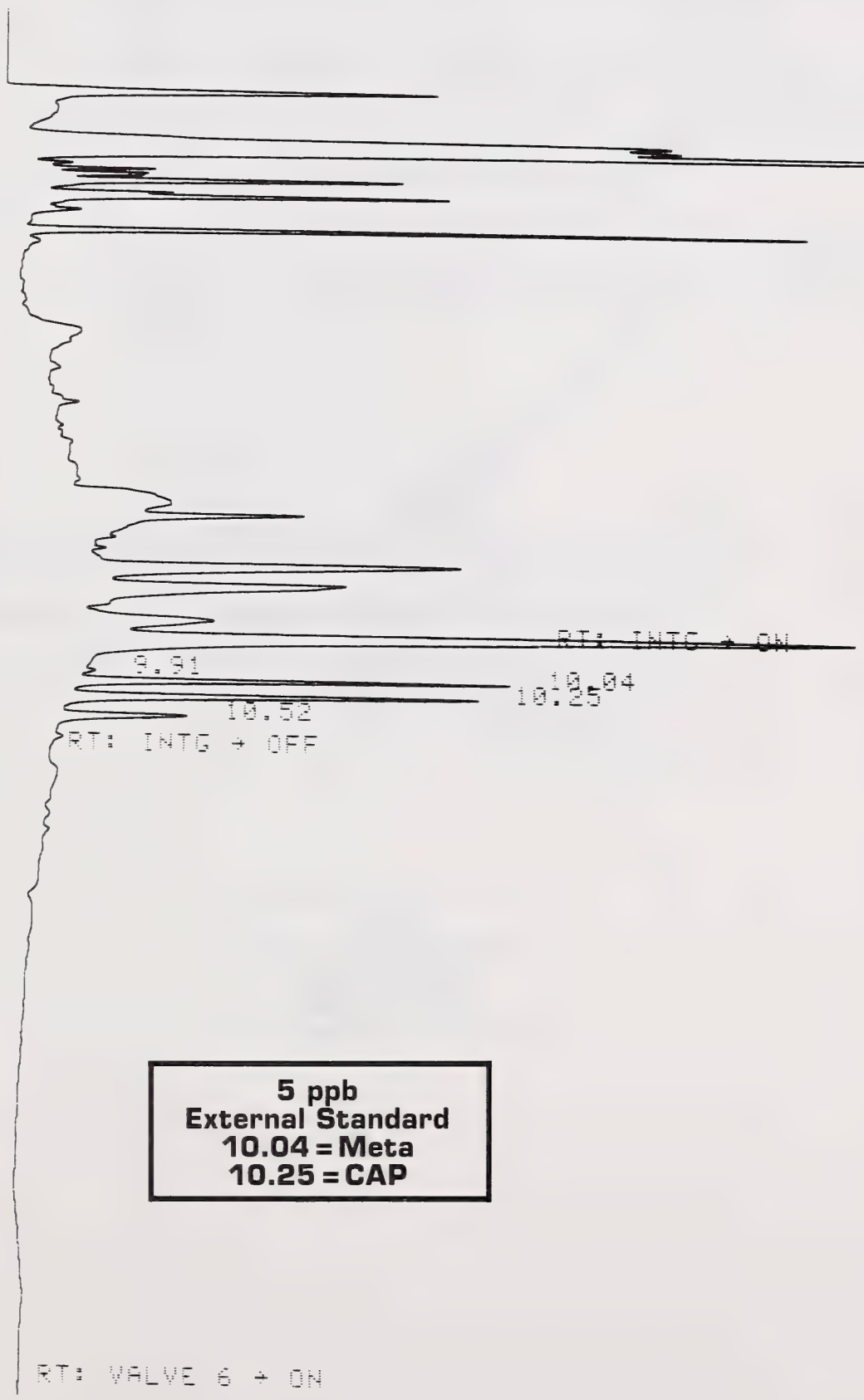
2. Interferences

No known interferences.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

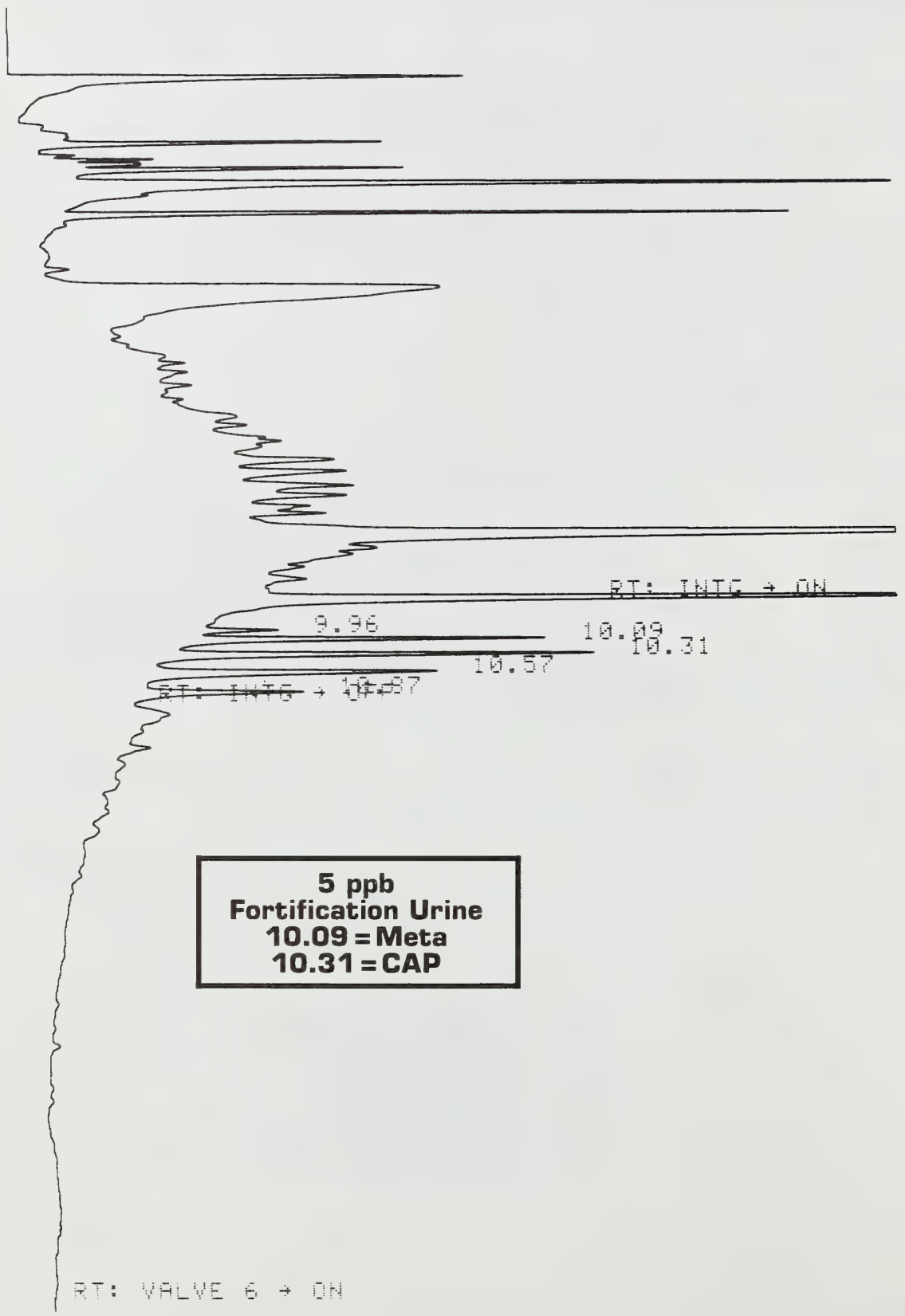
2. Sample Chromatograms



5 ppb
External Standard
10.04 = Meta
10.25 = CAP

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



5 ppb
Fortification Urine
10.09 = Meta
10.31 = CAP

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Metachloramphenicol is used as an internal standard for calculating chloramphenicol concentration. Proceed in the following manner to calculate linear regression calibration curves and chloramphenicol concentration.

   By an acceptable means, measure the peak height or peak area for each component in the 2.5 ppb, 5.0 ppb, and 10.0 ppb spiked samples that have been processed through the procedure. Calculate the peak height or peak area ratios for chloramphenicol by dividing its peak height or peak area by that of the metachloramphenicol peak height or peak area.

b. Using the ratios and associated ppb values, calculate a linear regression calibration curve by least squares computation as indicated in the Chemistry Quality Assurance Handbook Vol. II, Section 1.5.69. These calibration curves are then used to calculate values for additional samples from the sample set.

   $y = mx + b$, where

   m = slope

   b = intercept

   $$y = \frac{\text{chloramphenicol peak height or area}}{\text{metachloramphenicol peak height or area}}$$

   x = chloramphenicol concentration in ppb

Standard curve correlation coefficient ≥ 0.9945

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination and Confirmation of Chloramphenicol at Low Parts Per Billion in Calf Urine.

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat, and efficient fume hood.

3. Procedure Steps

CAUTION: Do not swallow, inhale, or absorb through skin any chemical, as complete toxicological properties for most chemicals are unknown.

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Enzyme: β-glucuronidase, type IXa	The toxicological properties have not been thoroughly investigated. May be harmful.	The analysis should be done under an efficient fume hood.
Solvents: Ethyl acetate, hexane, methanol, cyclohexane, chloroform.	Flammable, and the vapors are corrosive to the skin, eyes, and respiratory system. Chloroform is a carcinogen.	These solvents should be used only in an efficient fume hood, away from any electrical heating devices.
Analyte: Chloramphenicol Metachloramphenicol	Chloramphenicol has a wide range of possible adverse effects ranging from dermatitis, to embryo and fetal death, to death of adults. Chloramphenicol has been associated with inducing aplastic anemia. Those who survive aplastic anemia have a high incidence of developing acute leukemia. It is considered a carcinogen. The meta isomer is assumed to have similar toxicity.	Because the unique toxicity to humans has been well documented, the utmost care in handling chloramphenicol is recommended. See the material safety data sheet for information on how to handle any spills.
Derivatizing Reagents: 9:3:1 pyridine: hexamethyldisilazane: trimethylchorosilane	Flammable. This mixture can burn eyes, skin and may cause headache, nausea, dizziness, liver and kidney damage. Trimethylchlorosilane may be a possible carcinogen.	Use only in an efficient fume hood.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS (Continued)

4. Disposal Procedures	Organic solvents	See above	Store in the non-chlorinated solvent waste container until disposed of by the contractor or in-house specialist.
	Halogenated solvents	See above	Store in the chlorinated solvent waste container until disposed of by the contractor or in-house specialist.
	Chloramphenicol solutions	See above	Store separately in a well-marked container and hold for the contractor.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Chloramphenicol*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: CHLORAMPHENICOL

Analyst: _____ Matrix: _____

Date Started: _____ Date Completed: _____

Age of working standards:

Chloramphenicol: _____ Meta isomer: _____

Sample size: _____

Incubation time: At least 90 minutes

Sylon HTP volume: 200 μL ± 20 μL

Reaction temperature: 65 C ± 5 C

Final volume: 100 μL

Remarks:

Fortification Curve (ppb)	Retention Time-minutes meta CAP	Peak Height or Area Counts meta CAP	CAP:meta	Linear Regr
Control				b =
				m =
				r =
				s =

Sample Identification	Retention Time-minutes meta CAP	Peak Height or Area Counts meta CAP	CAP:meta	Amt Found (ppb)

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Compound	*Analytical Range (ppb)*	*Standard Curve Correlation Coefficient*	*Repeatability CV_o %*
Chloramphenicol	2.5 to 10.0	>0.9945	<20 at 5.0 ppb

2. Critical Control Points and Specifications

a. Intraset.

Record	*Acceptable Control*
β-glucuronidase solution	Prepare fresh daily.
Incubation time	Minimum incubation time is 90 min.
Incubation temperature	37° ± 2° C.
Sample size	5.0 mL ± 0.1 mL.
Working standard solution volume	Depends on fortification level.
Sylon HTP volume	200 μL ± 20 μL.
Reaction time	15 min ± 1 min.
Reaction temperature	65° ± 5° C.
Evaporation of excess Sylon HTP	See section E.1.aa.

b. Interset.

Age of working standard solution	One month.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each, on 3 separate days: 0 ng/μL, 0.125 ng/μL, 0.25 ng/μL, and 0.50 ng/μL, which are equivalent to the blank, 2.5 ppb, 5.0 ppb, and 10.0 ppb 5 mL fortified urine samples, with 1 mL urine approximately 1 g.

ii. Phase II: Fortified samples—4 levels, 6 replicates each. Same as above—over a minimum of three days, 24 total samples.

NOTE: Phase I and II may be performed concurrently.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division is required to commence official analyses.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

(d) Fortification curve correlation data.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptablility and Stability

a. Sample Storage: Samples are stable for six months if stored at −10° C.

b. Condition upon receipt: Cold.

6. Sample Set

a. A blank tissue.

b. Fortified blanks at 2.5, 5.0, and 10.0 ppb.

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 2.5 ppb.

b. Lowest reliable quantitation (LRQ): 5.0 ppb.

II. CONFIRMATORY METHOD

A. INTRODUCTION

Refer to Determinative Method, section A.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.1

2. Instrumentation

GC/MS: Extranuclear Model 275, quadrupole, with capillary inlet, splitless injection, fitted with an OV-1 25 m × 0.20 mm id fused silica crosslinked methyl silicone column, film thickness 0.33 μm (Hewlett-Packard #19091A-102, or equivalent).

C. REAGENTS

Refer to Determinative Method, section C.

D. STANDARDS

a. See Determinative Method, section D.

b. The same sample set which is used in the Determinative Method will be used by the mass spectrometer operator for confirmation. Only one standard is usually analyzed.

E. DERIVATIZATION

Sample Preparation

A suitable microliter volume of derivatized material obtained in the determinative procedure (see Determinative Method, section E) is injected into the GC/MS for confirmation.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions and parameters. These conditions are for the Extranuclear instrument described in section II.B.2 and are given as an example only. The analyst should optimize these parameters for the instrument being used.

Column: OV-1 Fused Silica Capillary, 25 m.

Injector: 230° C.

Temperature programming: 150° C with no initial hold, programmed to 300° C at 20° C/min. Final hold 10 min.

Transfer line temperature: 300° C.

Helium flow rate: 29 cm/sec, splitless injection.

b. Mass spectrometer conditions.

Detection: Enhanced negative ion chemical ionization (NICI).

Ionization gas: Isobutane or methane.

Source pressure: 1.0×10^{-5} T.

Source temperature: 260° C.

Mode of operation: SIM-NICI.

Dwell time: 10 millisec.

Calibration standard: PFTBA; maximize on ion 452.

Scan width: 0.2 amu.

Expected signal/noise of lowest intensity ion: 10/1.

Filament voltage: 300 eV.

Filament current: 1000 μA.

2. Required Samples for GC/MS Analysis

Set up instrument as described in section 1 above to monitor chloramphenicol and the internal standard metachloramphenicol. Inject 2 to 5 μL of the external standard and analyze results to verify that the system is functioning properly. If not, make any necessary adjustments in operating parameters or standard concentration, then reinject to verify performance. Inject 2 to 5 μL each of the confirmation sample, the recovery, and the tissue blank. Confirmatory analysis is required in all samples found positive by the determinative method.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

a. Retention time specification. Compare the retention time of the sample with the retention time of the fortified tissue. The times should match ± 5.0%. The following is an example only, as other retention times are accepetable.

Compound	*Expected Retention Time (min)**
Metachloramphenicol	8.2
Chloramphenicol	9.0

b. Ions 468, 466, 322, and 304 must be present. In samples with high levels of chloramphenicol, ions m/z 358 and 360 can also be monitored.

c. Compute at least the following three ratios for the standard, fortification, and the standard: 466/468, 322/466, and 304/466. For successful confirmation between levels 2.5 ppb and 10.0 ppb, the 466/468 and either the 322/466 or the 304/466 ion for the sample must agree within ± 20% of the ratio for fortified tissue. In samples with high levels of chloramphenicol, also compute ratios 358/466 and 358/360.

The ion ratios of the fortified standard must match the ion ratios of the sample by ± 20%. The following are expected ion ratios for the conditions cited.

466/468 = 1.43

322/466 = 0.49

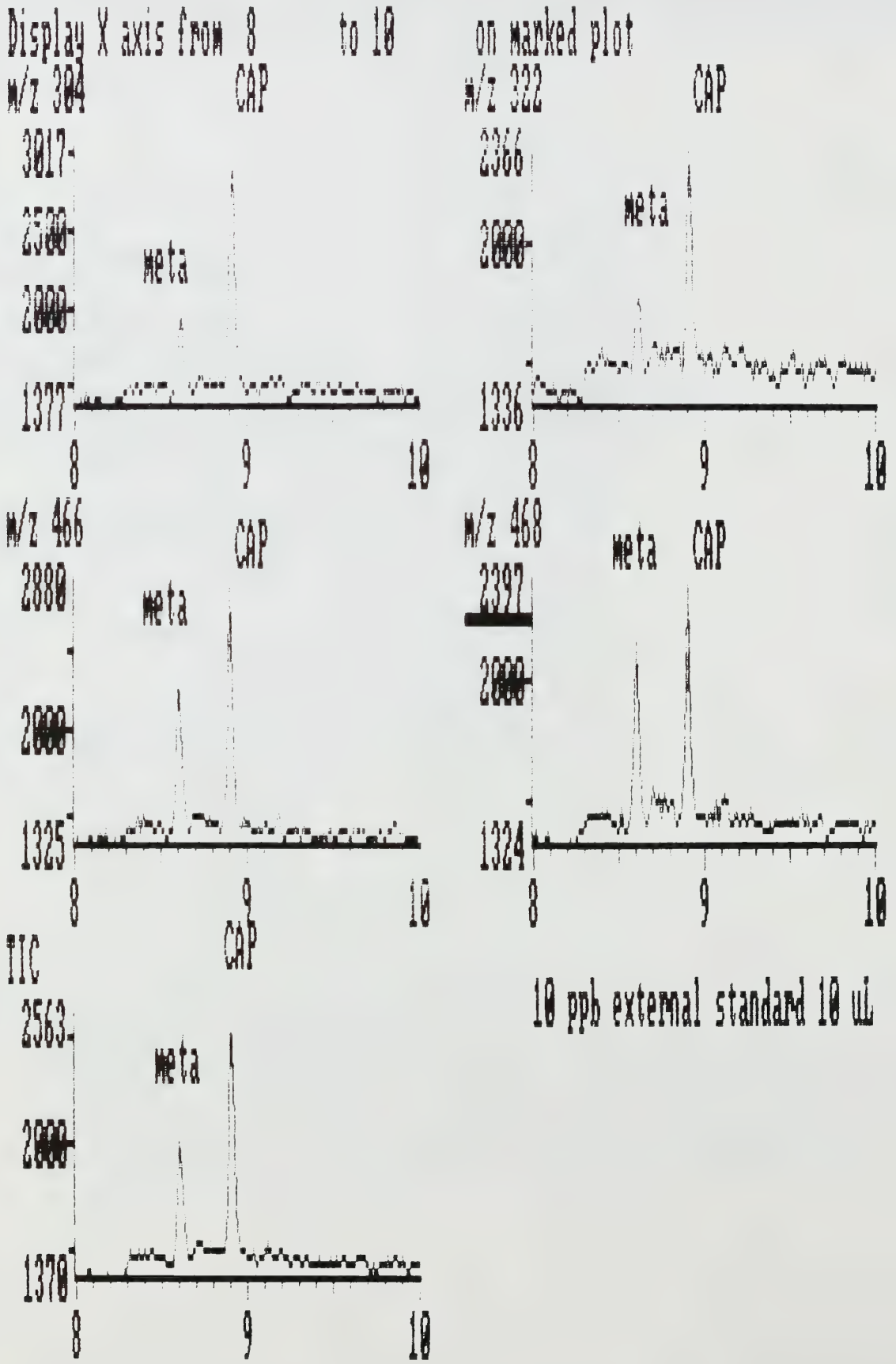
304/466 = 0.57

NOTE: The ion ratios are dependent on the source temperature.

II. CONFIRMATORY METHOD

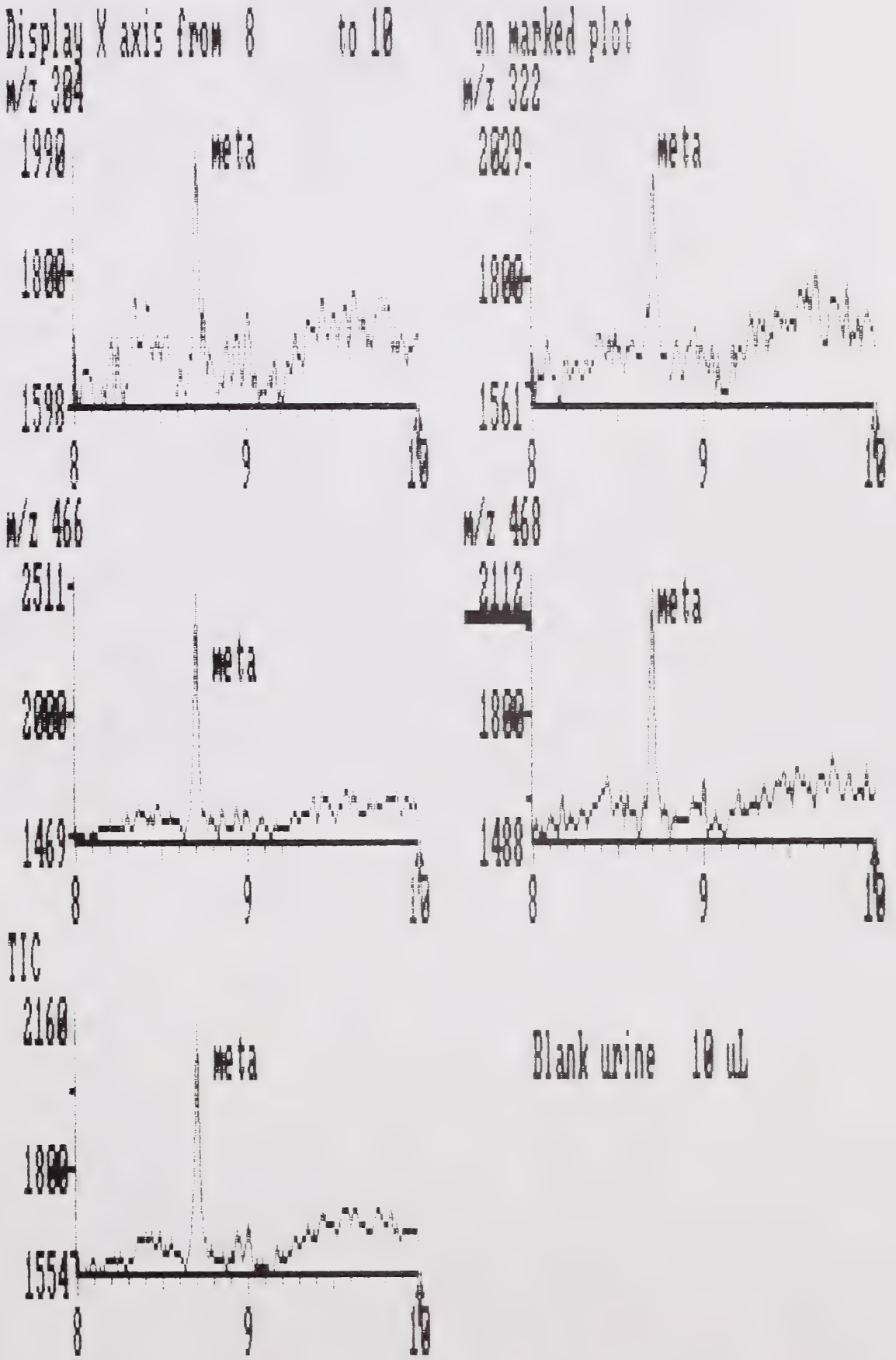
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

5. Chromatograms



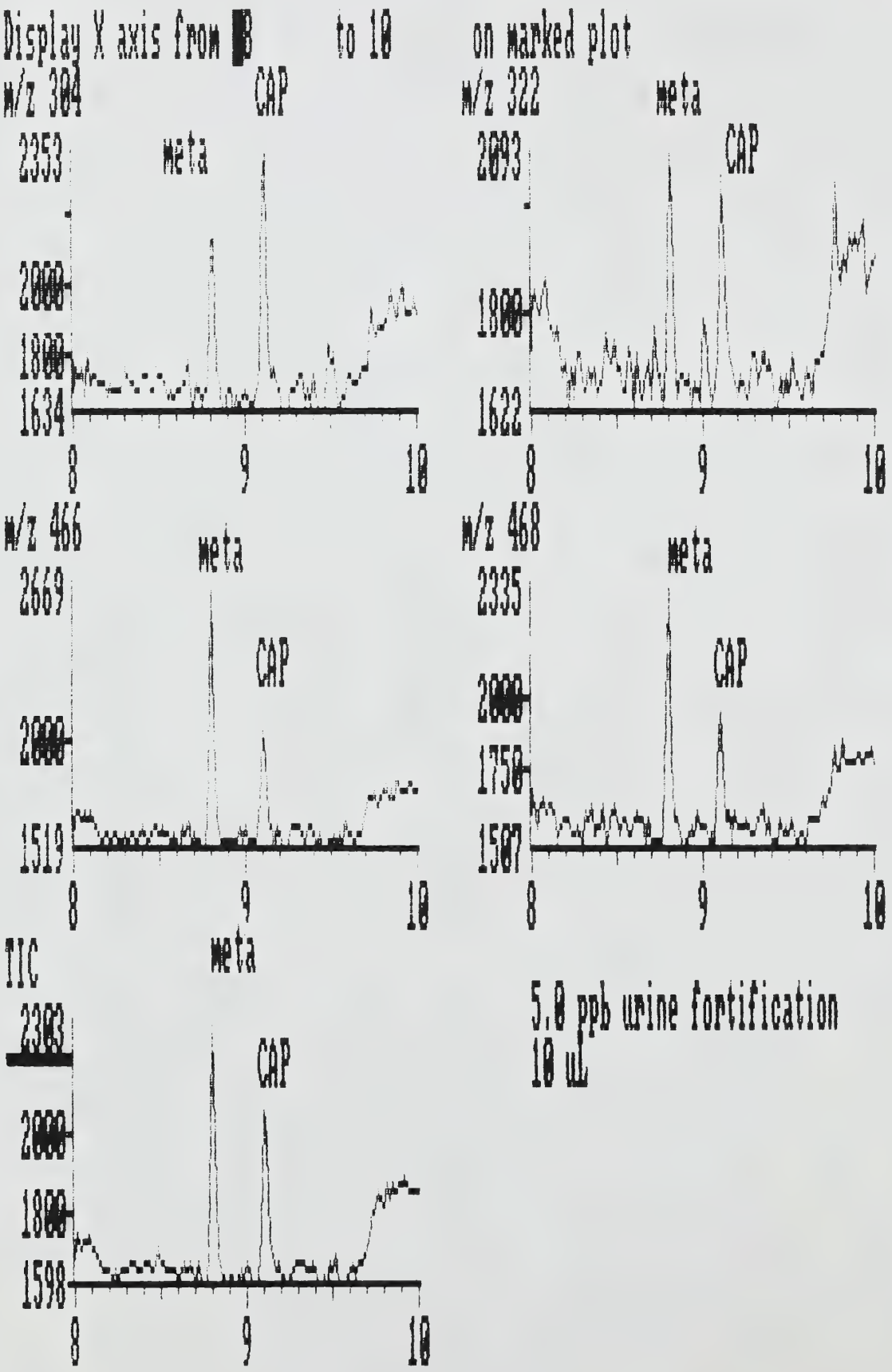
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

Refer to Determinative Method, section H.

I. WORKSHEET

The worksheet on the following page, *Mass Spectrometry Data Form*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte: _____ Tissue: _____

	R.T. (min) Base Peak	Ion Abundance (m/z)				Ion Ratios		
		_____	_____	_____	_____	_____	_____	_____
Blank								
Standard								
Fortified Tissue								
Sample #								

Concentration:

Standard: _____

Fortified tissue: _____

Sample: _____

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb.

b. No false negatives at 2.5 ppb.

2. Critical Control Points and Specifications

See Determinative Method, section J.2.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standards, show that the instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

i. No false positives or negatives.

ii. Must monitor at least 4 ions.

iii. Retention time of analyte peak must match the standard or fortified standard within ± 5.0%.

iv. Ion ratios for the 466/468 ion pair must match those of the 5.0 ppb fortified standard by ± 20%. In addition, one other ion ratio must match those of the 5.0 ppb fortified standard by ± 20%.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

b. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for :

i. All replicate findings.

ii. Control chart on differences between replicates.

iii. All percent recoveries.

iv. Fortification curve correlation data.

c. Acceptability criteria.

If values are unacceptable:

i. Stop all offical analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

---

5. Sample Acceptability and Stability

a. Sample storage: Samples are stable for six months if stored at $-10°$ C.

b. Condition upon receipt: Cold.

---

6. Set Construction

a. Standard.

b. Tissue Blank.

c. Tissue fortified at action levels.

d. Samples.

---

7. Sensitivity

a. Lowest detectable level (LDL): Not applicable.

b. Lowest reliable confirmation (LRC): 2.5 ppb.

CARBAMATES

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 6
C. Reagents and Solutions 10
D. Standards 11
E. Extraction Procedure 13
F. Analytical Quantitation 17
G. Calculations 20
H. Hazard Analysis 21
I. Worksheet 23
J. Quality Assurance Plan 27

IIA. Confirmatory Method

A. Introduction 31
B. Equipment 32
C. Reagents and Solutions 33
D. Standards 34
E. Sample Preparation and Cleanup 35
F. Analytical Confirmation Procedure 37
G. [Reserved]
H. Hazard Analysis 41
I. Worksheet 42
J. Quality Assurance Plan 45

IIB. Confirmatory Method

A. Introduction 47
B. Equipment 47
C. Reagents and Solutions 48
D. Standards 48
E. Sample Preparation and Cleanup 49
F. Analytical Confirmation Procedure 52
G. [Reserved]
H. Hazard Analysis 53
I. Worksheet 53
J. Quality Assurance Plan 57

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Carbamates are primarily systemic insecticides and acaracides but are also used extensively as soil treatments and as topical and knockdown agents for ectoparasites and other pests. Carbamates are cholinesterase inhibitors and are generally neurotoxic. Symptoms of toxicity include nausea, vomiting, diarrhea, and dyspnea.

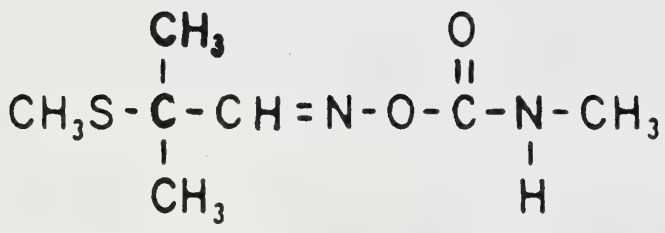
Carbamates are extracted from sample tissues using methylene chloride followed by gel permeation chromatography clean-up. The eluates containing carbamate residues are evaporated to dryness, reconstituted in methylene chloride, and further purified by passing through an aminopropyl Bond Elut cartridge and eluting with 1.5% methanol in methylene chloride, which can then be quantitated using LC equipped with a post-column OPA fluorescence detector.

2. Applicability

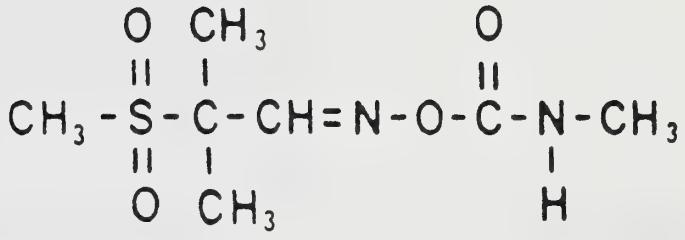
This method is applicable for carbamate residues analysis in liver of all species.

I. DETERMINATIVE METHOD

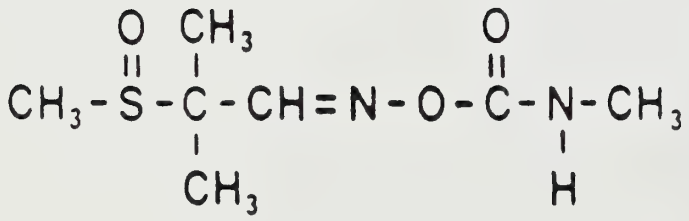
A. INTRODUCTION (Continued)



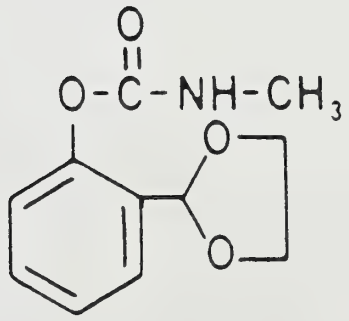
ALDICARB



ALDICARB SULFONE



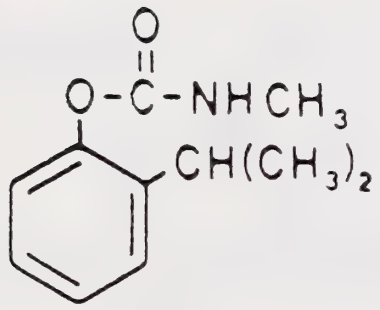
ALDICARB SULFOXIDE



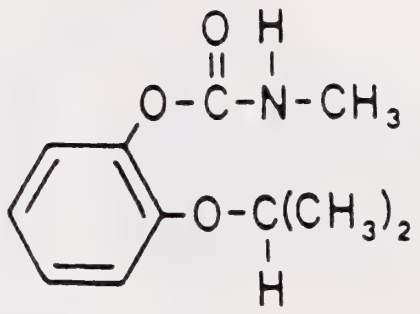
DIOXACARB

I. DETERMINATIVE METHOD

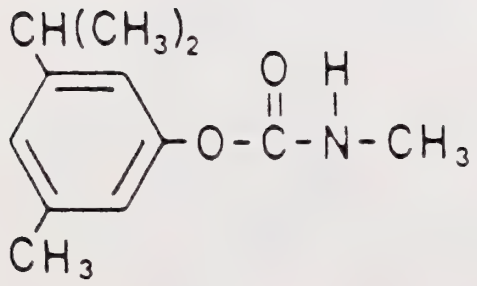
A. INTRODUCTION (Continued)



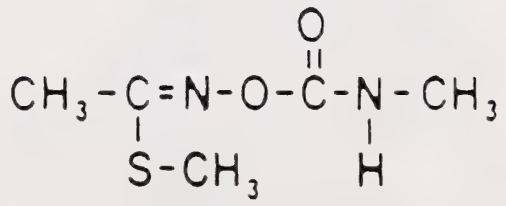
ISOPROCARB



PROPOXUR



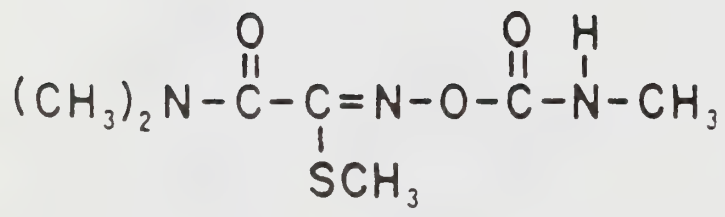
PROMECARB



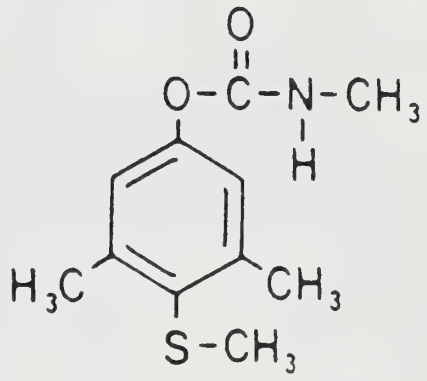
METHOMYL

I. DETERMINATIVE METHOD

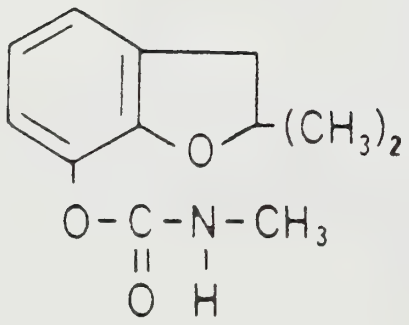
A. INTRODUCTION (Continued)



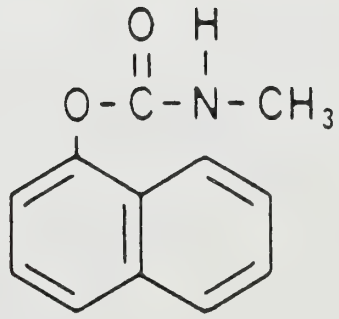
OXAMYL



METHIOCARB



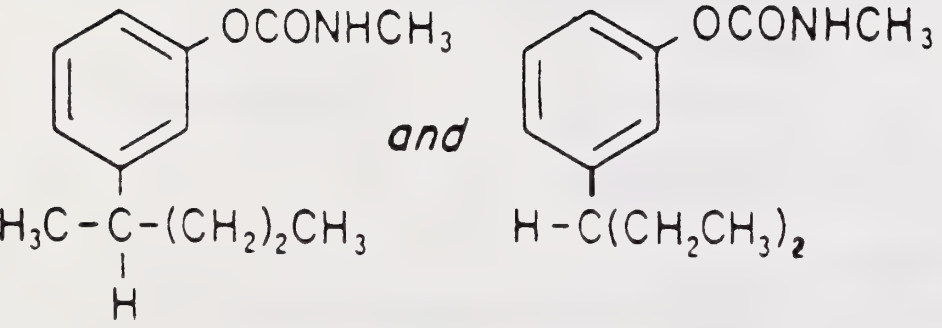
CARBOFURAN



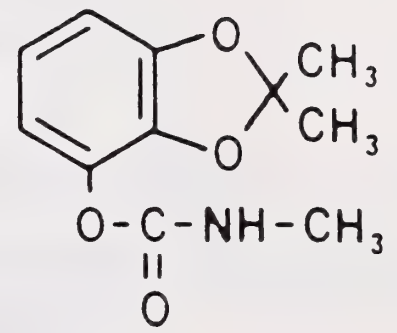
CARBARYL

I. DETERMINATIVE METHOD

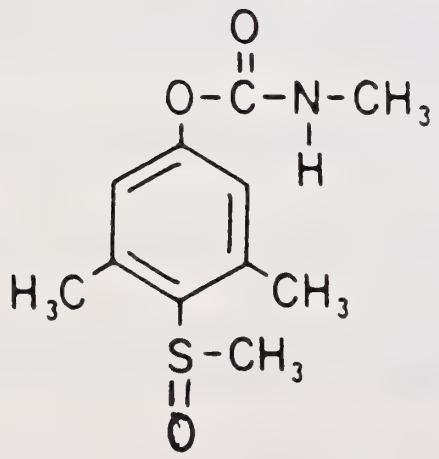
A. INTRODUCTION (Continued)



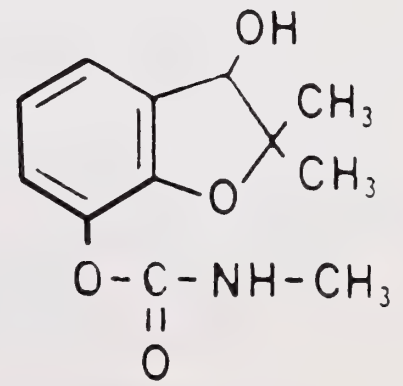
BUFENCARB



BENDIOCARB



METHIOCARB SULFOXIDE



3-HYDROXY CARBOFURAN

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: An equivalent may be substituted for all items listed below if necessary.

a. Blender, Laboratory: capacity 0.5 to 1 L (Waring Product Division, Dynamics Corporation of America, New Hartford, CT).

b. Homogenizer: Virtis ''45'' homogenizer (Virtis Co., Gardner, NY 12525).

c. Virtis flasks: capacity 500 mL (American Scientific Co.).

d. Top-loading balance: 1 div. = 0.1 g.

e. Buchner funnel: porcelain, 9.0 cm id.

f. Filter paper: Whatman #1 filter paper, 9.0 cm.

g. Powder funnel: Kimmax 58.

h. Filter paper: folded filter paper, 18.5 cm (Schleicher and Scheull, Inc., Keene, NH 03431).

i. Round-bottom flask: 24/40 joint boiling flask, Pyrex brand glass, 500 and 250 mL capacity.

j. Rotary evaporator: Buchi Model E (Brinkman Instrument, Inc.).

k. Centifuge tube: Conical, graduated, stopper size 13, Pyrex brand glass, 15 mL capacity, 17 mm od × 139 mm long (Curtin Matheson Scientific, Inc.).

l. Solvent-resistant filter unit: Millex-SR, 0.45 μm filter unit (Millipore Corp., Bedford, MA 01730).

m. Extraction cartridge: Aminopropyl Bond Elut cartridge—100 mg sorbent mass and 1 mL elution volume (Analytical International, Harbor City, CA 90710).

n. Evaporator—N-Evap: Model 111 or 112 (Organomation Associates, Inc., South Berlin, MA).

o. Maxi mix: Model M-167155 (Thermolyne Sybron Corporation, Dubuque, IA 52001).

p. Sample extraction apparatus: Acro LC 13 disposable filter assembly with 0.45 μm fluoropolymer membrane (Gelman Sciences, Inc., Ann Arbor, MI 48106).

q. Glass syringes: 100 and 250 μL fixed needle syringes and 1.0 and 10 mL capacity Luer tip syringes.

r. Limited volume insert: polypropylene inserts, 250 μl (Sun Brokers Inc., Wilmington, NC 28404).

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

s. pH meter.

t. Membrane filter: Magna nylon 66 membrane filter, 0.45 μm.

u. Solvent clarification kit: with membrane filters, but without pump and filtering flask (Waters Associates, Milford, MA 01757).

v. Volumetric flask, actinic: 500 and 25 mL.

2. Instrumentation

a. LC apparatus.

LC apparatus (see Figure 1 on the following page) must be capable of performing as described in LC operating parameters.

i. Liquid chromatograph: equipped with 2 pumps, Model 510, automated gradient controller, Model 680, Wisp 712 sample processor, and data module, Model 740 (Waters Associates).

ii. Detector: Spectroflow 980 programmable fluorescence detector, Part No. 9000-9801 (Kratos Analytical).

iii. Guard column: Guard Pak pre-column module, Part No. 80040, equipped with Bondapak C_{18} Guard Pak pre-column insert, Part No. 88070 (Waters Associates).

iv. Analytical column: 25 cm $\times$ 4.5 mm id, column containing 5 μm Zorbax C-8 spherical particles (DuPont Co.). Equivalent column should contain 5 or 6 μm spherical silica particles that have been bonded with a monofunctional octyl silane reagent to form a monomolecular bond. The outlet of the column is connected to the first tee of the reaction coil.

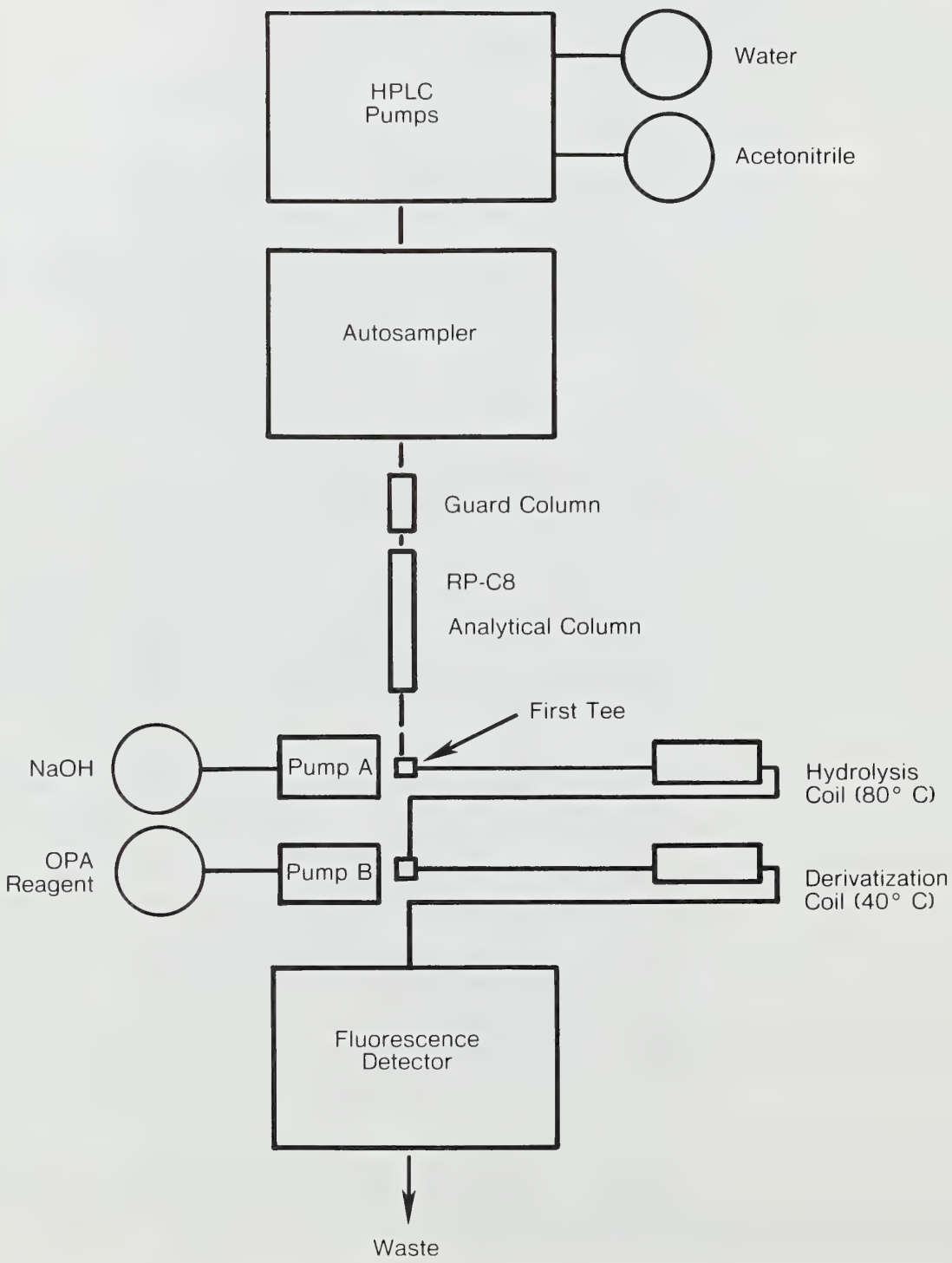
NOTE: The analytical column should be replaced when the resolution between the peaks of methiocarb sulfoxide and 3-hydroxy carbofuran methiocarb sulfoxide decreases to a critical point, i.e., when the resolution value (r) value drops below 1.0. Column performance can be monitored by adding 2-naphthol as an internal standard equivalent to 10 ppb: 40 μL of 2-naphthol (conc. 3.5 μg/mL methanol) to each 5, 10, and 20 ppb mixed standard and 20 μL to each sample extract, including recovery and blank prior to final dilution to appropriate volume for LC analysis, and observing the relative retention time of carbamates and 2-naphthol in the chromatogram. The peak of 2-naphthol appears between carbaryl and methiocarb peaks. The distortion of 2-naphthol peak or reduction of peak height in any chromatogram is an indication of column performance problem or LC problem.

v. Column heater: Model Delta Temp II (Dratos Analytical), equipped with 2 reaction coils, Part No. 1400-1324 URA 102, (Kratos Analytical), 1 mL volume each, and two tees connected in series with independent temperature contorls.

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

Figure 1 Schematic Diagram of LC Apparatus



I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

vi. NaOH pump: A pulse-free syringe pump, Model 314 metering pump, (ISCO, Inc., POB 5347, Lincoln, NE 68505) connected to the first tee of the reaction coil.

vii. OPA pump: same as NaOH pump except that it is connected to the second tee of the reaction coil and the outlet of the reaction coil is connected to the fluorescence detector.

viii. Connecting tubing: Use stainless steel tubing (1.6 mm od × 0.18 mm id) to connect injector, columns, tees, NaOH and OPA pumps, and detector.

b. Gel Permeation Chromatography System (GPC).

i. AutoPrep gel permeation chromatograph (Analytical Biochemistry Laboratories, Inc., P. O. Box 1091, Columbia, MO 56205), or equivalent with 60 × 2.5 cm id chromatographic tube packed with 60 g or about 48 cm bed length of BioBeads SX-3 resin (200-400 mesh). Methylene chloride:cyclohexane is used as the elution solvent. Flow rate should be calibrated to 5.0 mL/min, with the operating pressure at 7-10 psi.

ii. It is necessary to determine correct ''dump'' and ''collect'' times for the desired residues as a function of the pump flow rate. Before starting calibration, check the flow rate with SX-3 gel column connected and adjust the rate to 5.0 ± 0.3 mL/min (start pump ≥5 min before measuring rate to let the flow equilibrate). Rotate the ''load drum'' valve on the cabinet counterclockwise to ''load'' position. In a test tube, prepare a mixed standard of carbamate by evaporating 2 mL of ''working standard'' to dryness on a rotary evaporator with a bath temperature at 30° C, and reconstitute in 7.5 mL of methylene chloride: cyclohexane (1:1). Load the diluted standard onto the GPC using a 5 mL loop (2.67 μg of each standard). Dump 120 mL and collect 26 fractions of 5 mL in test tubes. Evaporate fractions to dryness and redissolve in 200 μL of methanol. Analyze by LC to determine which fractions contain carbamates and calculate the ''dump'' and ''collect'' times accordingly. Set the wash time at 4 min. Check calibration of GPC once every six months.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

NOTE: Solvent-quality grade: pesticide and distilled in glass.

a. Methanol.

b. Methylene chloride.

c. Cyclohexane.

d. Ethyl acetate.

e. LC acetonitrile: UV grade. Before use, filter through a 0.45 μm membrane filter and de-gas in a filtering flask by applying vacuum and slowly stirring solvent with a magnetic stirrer for 10 min.

f. LC water: Prepare by using Milli-Q water purification system. For use, de-gas H_2O as described above for LC acetonitrile.

g. NaOH solution.

   i. 5N NaOH: Weigh 200 g NaOH pellet in 1000 mL volumetric flask. Mix with water to dissolve and dilute to 1000 mL with LC water.

   ii. 0.05N NaOH: Pipet 10 mL NaOH into 1 L volumetric flask. Dilute to 1 L with LC water.

h. Potassium borate solution, 0.05M: Weigh 3.55 g ACS-grade potassium borate in a 2 L volumetric flask. Dissolve in about 1900 mL of LC water with a magnetic stirrer. Adjust pH to 10.5 with 5N aqueous solution of NaOH and dilute to volume.

i. Reaction solution: Weigh 70 mg of o-phthalaldehyde (OPA) (Fluoropa, Dionex Corp., Sunnyvale, CA 94086). Transfer to a 500 mL volumetric flask and add 5 mL of methanol. Stir using a magnetic stirrer to dissolve o-phthalaldehyde. Dilute to volume with 0.05M potassium borate solution. Filter through a 0.45 μm membrane filter into a 1 L filtering flask and de-gas. Add 0.5 mL of 2-mercaptoethanol (Aldrich Chemical Co., Inc.) while stirring slowly, preferably in a fume hood. Prepare just before use.

j. Sodium sulfate: Na_2SO_4, anhydrous powder, Baker Analytical Reagent (J. T. Baker, Inc.).

k. Methylene chloride: cyclohexane, 1:1 v/v.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Carbamate standards can be obtained from:

EPA/FDA Reference Standards
Environmental Monitoring Support Laboratory
Office of Research and Development
Cincinnati, OH 45268

b. Carbamate standards include the following:

i. Aldicarb sulfoxide.

ii. Aldicarb sulfone.

iii. Methomyl.

iv. Methiocarb sulfoxide.

v. 3-hydroxy carbofuran.

vi. Aldicarb.

vii. Carbofuran.

viii. Carbaryl.

ix. Methiocarb.

x. Bufencarb.

xi. Oxamyl.

xii. Dioxacarb.

xiii. Propoxur.

xiv. Bendiocarb.

xv. Isoprocarb.

xvi. Promecarb.

c. The external standard (2-naphthol) can be purchased from:

Aldrich Chemical Co.
940 West Saint Paul Avenue
Milwaukee, WI 53233
Telephone number: 800-255-3756

I. DETERMINATIVE METHOD

D. STANDARDS (Continued)

2. Preparation of Standards

a. Stock solution, 0.5 mg/mL: In each 25 mL actinic volumetric flask, dissolve 12.6 mg of each carbamate standard in about 15 mL of ethyl acetate and dilute to volume with ethyl acetate.

b. Working solution, 2.0 ng/μL: With a syringe, transfer 100 μL of each stock standard solution (equivalent to 50 μg) into a 25 mL actinic volumetric flask and dilute to volume with de-gassed methanol. Swirl several times to mix well. This solution is used in sample fortification and with further dilution, in a daily standard curve.

NOTE:

i. Since baseline separation between oxamyl and aldicarb sulfone and between carbofuran and bendiocarb is not possible in a mixed standard of all 16 carbamates, for the purpose of quantitation, it is necessary to run 2 separate sets of recoveries and standard curves: aldicarb sulfone and carbofuran in one set and the other 14 carbamates in the other set.

ii. Aldicarb is easily oxidized to aldicarb sulfoxide. Avoid incorporation of air in the working solution by not shaking vigorously.

iii. Simultaneous decrease of aldicarb peak height and increase of aldicarb sulfoxide peak height in a standard curve is the indication that it is time to discard old working solution.

c. LC standard curve solution: 175, 350, 600 ng/mL. Using a syringe, transfer 35, 70, and 140 μL of working solution from b above into three test tubes. Add methanol to each test tube to make a total volume of 400 μL. These solutions represent 5, 10, and 20 ppb respectively of each standard or 175, 350, and 600 ng per mL of each standard based on a 7.0 g sample.

3. Storage Conditions

All standards are to be kept in stoppered glass bottles at – 4° C.

4. Shelf Life Stability

a. Stock standards: 6 months.

b. Working standards: 2 months.

c. LC standards should be made fresh before use.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 21.0 ± 0.05 g of blended partially defrosted liver into a clean 500 mL Virtis flask.

NOTE: Blended frozen samples can be cut into small pieces and used without defrosting.

b. With each set of samples to be analyzed, weigh one control blank liver (previously analyzed and found to contain no carbamate) and one blank liver sample fortified with 105 µL working standard (which is equivalent to mid-level fortification or 10 ppb fortification).

c. Add 60 g of anhydrous Na_2SO_4 to the sample and mix with a spatula.

d. Add 200 mL of methylene chloride to the sample and again mix with a spatula.

e. Homogenize at medium speed for 2 min using Virtis homogenizer.

f. Transfer the clear filtrate onto a Buchner funnel containing a Whatman #1 filter paper attached to a 500 mL filtering flask. Add about 5.0 g of anhydrous Na_2SO_4 to facilitate the filtration process. Apply suction to complete the filtration process.

g. Add 100 mL of methylene chloride to the mixture of tissues and Na_2SO_4.

h. Homogenize for 1 min at medium speed.

i. Empty the Virtis flask onto the same Buchner funnel. Apply suction to complete the filtration.

j. Rinse the Virtis flask with 25 mL of methylene chloride and add onto the Buchner funnel. Apply suction to complete the filtration.

k. Refilter the above filtrate into a 500 mL round-bottom flask using a funnel and a folded filter paper containing about 2.0 g of anhydrous Na_2SO_4. Refiltration is necessary for homogeneous treatment of all samples; the first filtrate of some liver extracts quite often appears cloudy due to excess moisture.

l. Using a syringe, wash the filter paper with about 10 mL of methylene chloride.

m. Concentrate the combined extracts carefully to about 1-2 mL on a rotary evaporator with a water bath temperature at 30° C. (*Do not let the flask go dry.*)

n. Transfer the liquid portion into a 15 mL graduated test tube with a disposable pipet. First rinse the round-bottom flask with 1-2 mL of cyclohexane and transfer into the test tube. Transfer quantitatively the remaining residue in the flask by washing several times with small amounts of methylene chloride:cyclohexane (1:1) each time, using a disposable pipet. Take precaution to make the total volume in the test tube exactly 7.5 mL (equivalent to a 21 g sample).

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

o. Filter the contents of the test tube directly into the 10 mL loading syringe of the GPC using a 10 mL Luer-tip syringe attached to a solvent-resistant filter unit.

p. Load the sample onto the GPC using a 5 mL loop (equivalent to 14 g sample).

q. Based on the results of the GPC calibration procedure, dump the appropriate volume of the GPC eluate and collect the sample residue fraction in a 250 mL round-bottom flask.

r. Evaporate the solvent to dryness on a rotary evaporator with a water bath at 30° C.

s. Transfer the residue quantitatively with a disposable pipet into a 15 mL graduated test tube by washing the flask with 10 mL of methylene chloride.

t. Concentrate the washing in the test tube to about 1 mL on a N-Evap with a water bath at 30° C.

u. Reconstitute to exactly 2 mL with methylene chloride.

v. Transfer 1.0 mL of the residue solution (equivalent to 7.0 g of sample) on a pre-washed Bond Elut cartridge (cartridge prewashed with 1 mL of methylene chloride).

w. Collect the eluate into a 15 mL graduated test tube.

x. Elute carbamate residues with 3.0 to 5.0 mL 1.5% methanol in methylene chloride.

NOTE: Experience with aminopropyl Bond Elut cartidge showed that the activity of the packing material occasionally differs from batch to batch. If the recovery of aldicarb sulfoxide drops below 80% in two consective runs, the final wash volume in step x of 1.5% methanol in methylene chloride should be carefully increased as much as 5 mL to obtain 80 to 90% recovery of aldicarb sulfoxide.

y. Evaporate the combined eluates on the N-Evap to dryness with a water bath at 30° C. *Do not overdry.*

z. Using a syringe, add 200 μL of methanol and vortex for 5 sec (conc. 350 ng/mL of 10 ppb recovery).

aa. Filter through a fluoropolymer or a nylon 66 membrane filter assembly into a limited volume insert and stopper for HPLC analysis.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary

Determination of N-Methylcarbamate Pesticides in Liver by Liquid Chromatography

STEPS:

21.0 g sample in 500 mL Virtis flask

b. For recovery, add 210 ng mixed standard (10 ppb) to a ''blank'' sample. Process one ''blank'' sample for each set.

c. Add 60 g anhydrous sodium sulfate and mix.

d. Add 200 mL methylene chloride.

e. Homogenize for 2 min.

f. Filter top clear solution using a Buchner funnel containing 5 g anhydrous Na_2SO_4 attached to a 500 mL filtering flask.**

Residue in Virtis flask.

g. Add 100 mL methylene chloride.

h. Homogenize for 1 min.

i . Empty contents onto the Buchner funnel of step f.

j . Rinse Virtis flask with 25 mL $MeCl_2$ and add onto Buchner funnel.

Filtrate in 500 mL filtering flask. ** (Step f)

k-l. Refilter using a funnel containing a folded filter paper with 2 g anhydrous Na_2SO_4 into a

500 mL round-bottom flask

m. Concentrate to ca 2 mL at 30° C.

n. Transfer quantitatively washing with 1 + 1 cyclohexane/ $MeCl_2$ to a 15 mL graduated test tube. Make *total volume exactly*

7.5 mL

o-q. Filter into a GPC loading syringe, load GPC, and carry out GPC separation. Collect residues in 250 mL RBF.

Continued on CBM-16.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

STEPS:

250 mL RBF

r-u. Evaporate transfer quantitiatively into a 15 mL tube and concentrate to ca 1 mL and reconstitute with $MeCl_2$ to 2 mL.

v-w. Transfer 1 mL onto a prewashed Bond Elut cartridge.

x. Elute carbamate residues with 3-5 mL 1-5% MeOH in $MeCl_2$ into

Test tube

y-aa. Evaporate, reconstitute with MeOH to 200 μL, and filter into a

Mini vial for LC analysis.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

The following parameters may change depending upon daily instrument variation:

i.	NaOH pump:	0.05N NaOH solution.
	Flow rate:	0.27 ± 0.02 mL/min.
	NaOH coil temperature:	80° C.
ii.	OPA pump:	Reaction solution (See section C. i).
	Flow rate:	0.27 ± 0.02 mL/min.
	NaOH coil temperature:	40° C.
iii.	Mobile phase	
	Initial condition:	50% acetonitrile in water.
	Flow rate:	1.50 ± 0.02 mL/min.
iv.	Wavelength settings:	
	Excitation:	350 nm
	Emission:	418 nm
v.	Injection volume:	20-30 μL of 10 ppb standard

2. Analysis

Set up the LC system as stated in section F.1. Equilibrate it before use by flushing with 12% acetonitrile in water solution for 20 min. Inject 20 to 30 μL of 10 ppb standard (350 ng/mL) and begin 30 min linear gradient to 70% actonitrile solution in water; another 1 min to 80% actonitrile in water; hold for 8 min; then equilibrate at the initial condition for 10 min. Adjust sensitivity on data module so that 10 to 12 ng carbofuran produces 50 ± 5% full-scale response. Baseline noise should be <2%. LC is now ready for sample analysis.

NOTE: Reapeatability of the method depends largely on the uniform pumping capabilities of all four pumps. As long as all solvents, including reaction solutions, are de-gassed, the pump does not give any problems. Prior to starting the system, check the flow rates of all four pumps and adjust to meet the flow rate specifications. At the beginning, if peak shifting, peak missing, or less sharp initial peak(s) are observed, allow more time to equilibrate the system. The appearance of flat noninterfering peak, noisy baseline, or baseline shifting are an indication of contaminated solvents or old solvents. Freshly prepared mobile phase, including reaction solution, will eliminate these problems.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. Retention Times

Compound Name	*Approximate retention time (min)*
Aldicarb sulfoxide	6.5
Oxamyl	9.3
Aldicarb sulfone	9.7
Methomyl	10.6
Methiocarb sulfoxide	12.9
3-Hydroxycarbofuran	13.8
Dioxacarb	15.3
Aldicarb	18.7
Propoxur	22.1
Carbofuran	22.5
Bendiocarb	22.7
Carbaryl	23.7
Isoprocarb	25.6
Methiocarb	28.1
Promecarb	29.7
Bufencarb	32.2

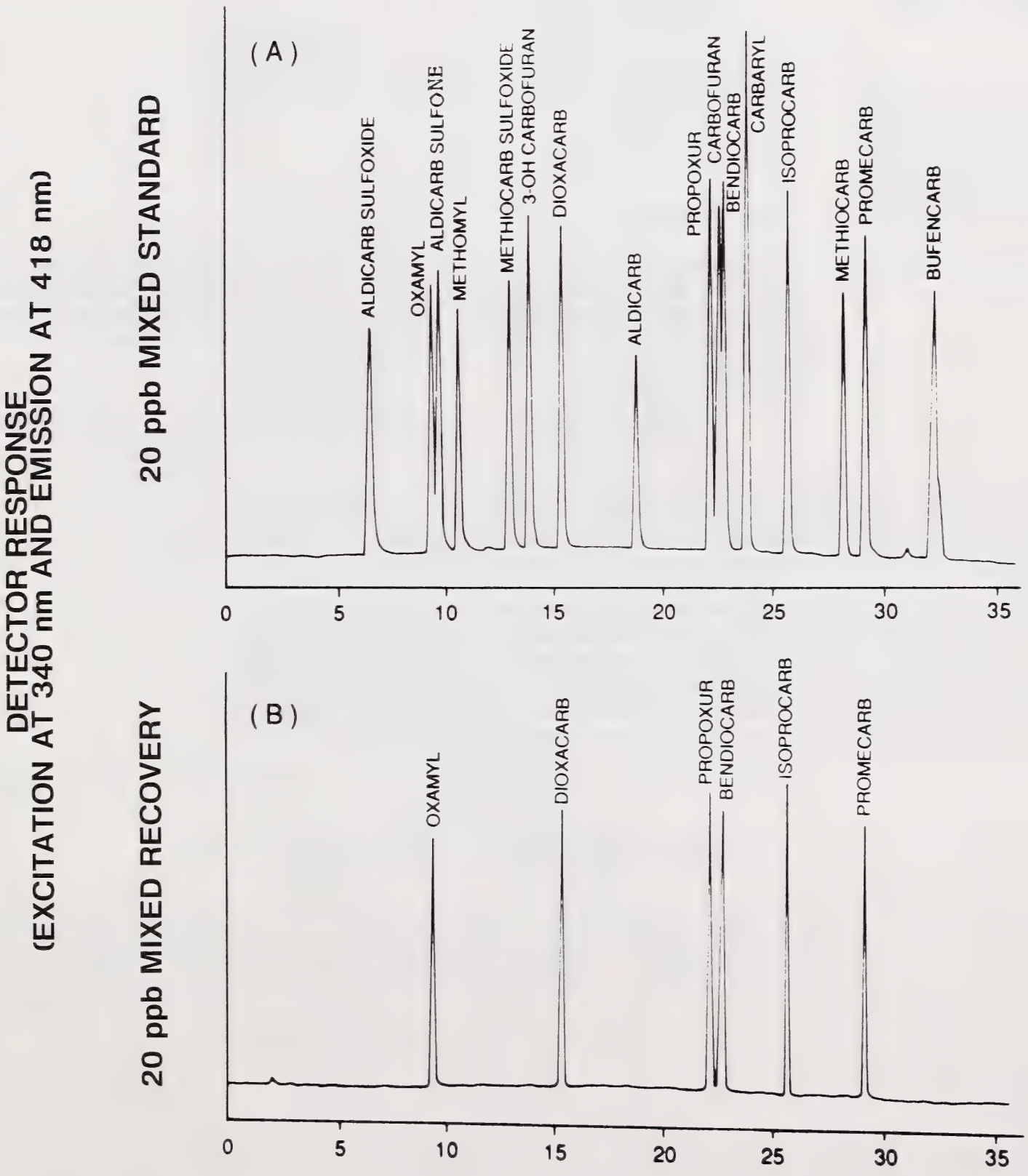
NOTE: Retention may vary from column to column.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

Figure 2

LC chromatograms of (A) mixed standards of 16 carbamates at the highest level used in the method and (B) mixed recoveries of 6 carbamates at the highest level used in the method. Post-column OPA fluorescence, excitation, and emission at 340 and 418 nm respectively.



I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

a. Establish the standard curve by injecting 20-30 μL of the LC standard solution, section D, item 2.c. Using peak heights and concentrations in ppb of standards, construct a linear standard curve for each compound based on the formula:

y = mx + b, where

y = the peak height

x = the concentration in ppb

m = the slope

b = the y intercept

The correlation coefficient should be $\geq$ 0.9900.

b. Calculate the recovery of the fortified sample and correct sample results for the running average recovery of the last 10 acceptable recoveries. Reanalyze violative samples. ''Acceptable recovery'' is defined as plus or minus 2 standard deviations from statistical mean recovery.

NOTE:

Standard curve (ng/ml)	*Standard curve (ppb)**
175	5
350	10
600	20

Correlation coefficient should be $\geq$ 0.9900.

Standard curve correlation coefficient $\geq$ 0.9945

*Calculated based on 7.0 g of sample weight:

$$\text{Sample weight: } \frac{5 \text{ mL (step E.1.p)}}{\text{(7.0 g) (step E.n)}} \times 0.5 \text{ (step E.1.u-v)} \times 21.0 \text{ g}$$

$$\text{ppb actual: } \frac{\text{ppb found from standard curve}}{\text{\% recovery of fortified tissue}} \times 100$$

2. References

Ali, M. Sher. Determination of N-Methylcarbmate Pesticides in Liver by Liquid Chromatography. *J. Assoc. Off. Anal Chem.* 72, 586-591. (1989).

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Determination and Confirmation of Carbamates in Tissues.

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Carbamate standards	Refer to NOTE below.	Standard preparation and sample extraction must be carried out in a well-ventilated hood and away from any source of ignition.
Methanol Methylene chloride Ethyl acetate Hexane Pentane Cyclohexane Acetonitrile 2-Mercaptoethanol	These solvents can produce toxic effects through exposure to skin, eyes, or respiratory system, and ingestion. All but dichloromethane are flammable.	Same as above.

NOTE: Carbamates, cholinesterase-inhibiting pesticides cause reversible carbamylation of the acetyl cholinesterase enzyme of tissues, allowing accumulation of acetylcholine at cholinergic neuroeffector junctions (muscarinic effects), and at skelatal muscle myoneural junctions and in autonomic ganglia (nicotinic effects). Poison also impairs central nervous system function. The carbamyl-enzyme combination dissociates more readily than the phosphorylated enzyme produced by organophosphated insecticides. The liabiltiy tends to mitigate the toxicity of carbamates, but also limits the usefulness of blood enzyme measurements in diagnosis of poisoning. Carbamates absorbed by inhalation, ingestion, and dermal penetration. They are actively metabolized by the liver, and the degradation products are excreted by the liver and kidney.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS (Continued)

4. Disposal Procedures	Solvents and standard solutions	See above.	Dispose of in proper waste drum for chlorinated vs. nonchlorinated solvents.
	Inorganic solution	See above.	Flush down in sink followed by lots of water.
	Sep-pak columns saturated with methanol and extracted residues.	See above.	Allow columns to dry in fume hood. Collect in plastic and discard.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Carbamates*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

CARBAMATE CALCULATIONS AND WORKSHEET

Analyst: _____ Date: _____

CARBAMATE CALIBRATION CURVE DATA

CONC PPB	CARBAMATE RESPONSE (HEIGHT)															
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
0																
5																
10																
20																

CARBAMATE LINEAR REGRESSION TABLE

STATISTIC	STATISTIC VALUE BY CARBAMATE															
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
r^2																
r																
m																
b																
s																

PEAK HEIGHT DATA FOR CARBAMATE SAMPLES

SAMPLE NAME	CHROMATOGRAPHIC RESPONSE (AREA/HEIGHT)															
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
BLANK																
RECOVERY																
CHECK																

CONCENTRATIONS FOUND (PPB) FOR CARBAMATE SAMPLES

SAMPLE NAME	CONCENTRATION IN PARTS PER BILLION															
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
BLANK																
RECOVERY																
CHECK																

PERCENTAGE RECOVERY FOR CARBAMATE CONTROLS

CONTROL (PPB)	PERCENTAGE RECOVERY															
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)
10																
HIGH																
LOW																

CARBAMATE IDENTIFICATION:

(1) ALIDCARB SULFOXIDE
(2) OXAMYL
(3) ALDICARB SULFONE
(4) METHOMYL
(5) METHIOCARB SULFOXIDE
(6) 3-HYDROXYCARBOFURAN
(7) DIOXICARB
(8) ALDICARB
(9) PROPOXUR
(10) CARBOFURAN
(11) BENDOCARB
(12) CARBARYL
(13) ISOPROCARB
(14) METHIOCARB
(15) PROMECARB
(16) BUFENCARB

DETECTION CONDITIONS:
Fluorescence Detection
Excitation 340 nm
Emission 418 nm

CHROMATOGRAPHIC CONDITIONS:

Analytical Column—Zorbax C-8
(25 cm × 4.6 mm id, micron)
Guard Column—Guard-Pak C-18
Flow Rate—1.5 mL/min
Column Temperature—Ambient
Gradient Elution—
(Acetonitrile and water)
(Initial—12% acetonitrile)
(30 min linear to 70% acetonitrile)
(1 min linear to 80% acetonitrile)
(8 min hold at 80% acetonitrile)
(10 min reequilibration to initial)

CONTROL POINTS:

E.1.a—Sample weight: 21.0 g
E.1.b—10 ppb recovery: Add 210 ng mixed stds
E.1.d, E.1.g—Methylene chloride volume: 200 & 100 mL
E.1.e, E.1.h—Blend time: 2 & 1 min
E.1.m, E.1.r, E.1.y—Water bath temperature: 30° C

ANALYST'S INITIALS _____

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Compound	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability CV_o %*
Aldicarb sulfoxide	5 – 20	50 – 100	13
Oxamyl	5 – 20	60 – 120	16
Aldicarb sulfone	5 – 20	60 – 100	13
Methomyl	5 – 20	40 – 100	20
Methiocarb sulfoxide	5 – 20	65 – 120	10
3-Hydroxycarbofuran	5 – 20	50 – 105	20
Dioxacarb	5 – 20	60 – 125	15
Aldicarb	5 – 20	60 – 105	15
Propoxur	5 – 20	60 – 120	15
Carbofuran	5 – 20	60 – 120	14
Bendiocarb	5 – 20	55 – 120	15
Carbaryl	5 – 20	60 – 100	14
Isoprocarb	5 – 20	55 – 115	15
Methiocarb	5 – 20	60 – 105	15
Promecarb	5 – 20	60 – 115	15
Bufencarb	5 – 20	60 – 100	14

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
	Sample weight	21.0 ± 0.05 g
	Filter and de-gas LC acetonitrile and LC water	Yes
	Filter and de-gas OPA solution before adding 2-mercaptoethanol	Yes
	Filter extract before GPC	yes
	Evaporation	Do not let dry up.
	Evaporation	Do not let over-dry.
	Use freshly prepared mobile phase and reaction solutions.	Yes

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Analyst is required to run 6 sets of analyses of mixed standards at 0, 5, 10, and 20 ppb, one set a day for standard curve generation.

(a) 0 ppb
(b) 5 ppb
(c) 10 ppb
(d) 20 ppb

ii. Phase II: Fortified samples—Run 6 sets of recoveries (2 sets for beef liver, 2 sets for swine liver, 2 sets for duck liver) using 0, 5, 10, and 20 ppb fortification of mixed standards on 6 different days. Calculate the recovery of fortified samples and tabulate results. The range of recoveries should stay within the acceptable recovery range as shown in the performance standard.

Data from standards and fortified samples are reviewed by the supervisor.

NOTE: Phase I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) A total of 8 blank liver samples will be fortified by the supervisor using different concentrations of mixed standards ranging from 7 to 20 ppb. The analyst will be required to analyze these check samples using a blank and a 10 ppb level fortified recovery and tabulate the results.

(b) The supervisor will report the analytical findings along with fortified amounts through the Laboratory staff officer to the Chemistry Division.

Notification from Chemistry Division is required to commence official analyses.

b. Acceptability criteria.

Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. At least one check sample biweekly per analyst containing at least one of the carbamates and using different species. At least one out of four of the positive samples is to be confirmed by mass spectrometry.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on percent differences between replicates.

(c) All percent recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptablility and Stability

a. Matrix: Liver.

b. Sample receipt size: minimum: 50 g.

c. Condition upon receipt: Cold (<10° C).

d. Sample storage: See table on following page.

e. Sample stability: See table on following page.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

Table of Carbamate Stability

Analyte/Species	*% Loss*	*Time I*	*% Loss*	*Time II*
Methomyl/Beef	94	1 day	100	0.5 month
Aldicarb Sulfoxide/Duck	50	1 day	100	3 months
Aldicarb Sulfoxide/Beef	50	0.5 month	65	6 months
Aldicarb Sulfone/Duck	50	1.5 months	100	6 months
Aldicarb Sulfone/Beef	50	0.5 months	65	6 months
Aldicarb/Duck	50	1 month	100	5 months
Aldicarb/Beef	50	1.5 months	100	6 months
Carbaryl/Chicken	20	4 months	60	6 months
Carbaryl/Beef	40	4 months	60	6 months
Bufencarb/Beef*	30	3 months	50	6 months
Carbofuran/Beef	20	3 months	40	6 months
3-OH Carbofuran/Beef	10	3 months	10	6 months
Methiocarb Sulfox/Beef*	60	3 months	56	6 months
Methiocarb/Beef**				
Oxamyl/Beef	97	1 day	100	0.5 month
Dioxacarb/Beef	50	0.5 month	100	6 months
Bendiocarb/Beef	60	1 month	92	6 months
Isoprocarb/Beef	55	3 months	65	6 months
Promecarb/Beef	50	0.5 month	100	6 months

* Anomaly exists; levels temporarily increased during the study period, possibly due to enzymatic activity.

** Methiocarb levels rose to approximately 1.5 times the original value and remained at that level for the duration of the study.

The samples used for this study were prepared at room temperature and stored in a freezer at 4° C for scheduled analysis.

6. Sample Set

a. A blank tissue.

b. Fortified blank tissue at 10 ppb.

c. 6 – 10 additional samples per set.

7. Sensitivity

a. Lowest detectable level (LDL): 5 ppb.

b. Lowest reliable quantitation (LRQ): 10 ppb.

c. Minimum proficiency level (MPL): Not determined.

IIA. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory	This method is used to confirm residues of bendiocarb, bufencarb, carbaryl, carbofuran, 3-hydroxycarbofuran, dioxacarb, isoprocarb, methiocarb, promecarb, and proxopur. The extract obtained from the determinative method is further purified using reverse phase SPE chromatography, then derivaitized with heptafluorobutyric anhydride. The derivatives are analyzed using GC/EI-MS in the SIM mode.
2. Applicability	Refer to Determinative Method, section A. 2.

IIA. CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Refer to Determinative Method, section B. 1. Equivalent equipment may be substituted unless noted.

a. C-18 Sep-Pak cartridges: Waters #51910.

NOTE: Verify elution parameters for every new lot of Sep-Paks used using 3-hydroxycarbofuran and bufencarb standards. Apply standards (100 ng each) to Sep-Pak and elute as in E.1.c through E.1.g below, collecting wash and elution fractions. Concentrate both fractions and inject on HPLC (refer to Determinative Method). Presence of 3-hydroxycarbofuran in the 10% wash means that the methanol concentration in the wash solution must be decreased. Poor recoveries of bufencarb means that the amount of 70% methanol eluant used must be increased, or a higher methanol concentration used.

b. Vortex mixer: Thermolyne Maxi-Mix.

c. Centrifuge tubes: Graduated 15 mL tubes with glass stoppers. Pyrex #8084.

d. Disposable pipette, Pasteur type, 5 1/4″ and 9″ lengths. Kimble #72050.

e. Micro reaction vials, 1 mL capacity, with Teflon liner and screw cap. Kimble #60710.

f. Round-bottom flask, 50 or 100 mL with 24/40 ground glass joint.

g. Glass syringe, 10 mL capacity, for eluting Sep-Paks. Sep-Pak cartridge rack or equivalent device for eluting columns using vacuum or positive displacement may also be used.

2. Instrumentation

a. Mass spectrometer: Hewlett-Packard 5970 Mass Selective Detector with capillary direct interface.

b. Gas chromatograph: Hewlett-Packard 5890 equipped with capillary split/splitless injector.

c. Column: 12 m × 0.2 mm × 0.33 micron HP-1 (bonded methyl silicone).

IIA. CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

NOTE: Refer to Determinative Method, section C, for reagents needed for sample extraction.

a. Methanol.

b. Dichloromethane (DCM).

c. Ethyl acetate.

d. Iso-octane.

e. Pentane (petroleum ether may be substituted).

f. Heptafluorobutyric anhydride, 98%. Aldrich Chemical.

g. Sodium sulfate, anhydrous, reagent grade.

NOTE: All solvents pesticide quality (Burdick and Jackson, distilled-in-glass).

h. Sodium sulfate, 5%. Dissolve 5 g sodium sulfate in distilled water, adjusting volume to 100 mL.

i. Methanol/water, 10%. Dilute 10 mL methanol to 100 mL with distilled water.

j. Methanol/water, 70%. Dilute 70 mL methanol to 100 mL with distilled water.

k. Potassium phosphate dibasic, 0.1 molar. Dilute 22.8 g $K_2HPO_4(3H_2O)$ to 1 L with distilled water.

IIA. CONFIRMATORY METHOD

D. STANDARDS

Preparation of Standards

a. Stock standards. Refer to Determinative Method, section D.1-2, for source and preparation of stock standards.

b. Working standards (2 μg/mL). Transfer 100 μL of each stock standard into a separate 25 mL volumetric flask, and dilute to volume with dichloromethane.

c. Derivatization standards. Prepare an external standard for derivatization by adding 100 μL (200 ng) of all appropriate working standards to a 1 mL reaction vial, taking the mixture just to dryness on an N-Evap, and reconstituting with 0.25 mL ethyl acetate. Confirmation of methiocarb requires preparation of a second standard containing 10 μg (20 μL of 0.5 mg/mL stock solution) in the reaction vial.

NOTE: Methiocarb can oxidize and form a second derivative under the conditions used in this method. In the presence of sample matrix, this secondary reaction is slowed to the point where the concentration of the second derivative is quite low. In the absence of enough co-extracted materials, such as with very clean samples and standards, the reaction can proceed to the point where only the second derivative can be found. This is particularly true when small amounts (50-150 ng) of analyte are derivatized. Use of a high standard concentration (10 μg) retards the rate of the secondary reactions and provides enough of the mono derivative to make confirmation possible using the external standard.

IIA. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP

1. Extraction

a. Weigh sample as described in Determinative Method, section E.1.a. Fortify the sample with an appropriate surrogate such as carbaryl or carbofuran, at a concentration equivalent to that of the compound of interest. Extract and clean up the sample as described in Determinative Method, section E.1.b through E.1.t. Ignore step u and transfer the entire sample onto the Bond-Elut cartridge in step v. Proceed normally through step y.

b. Redissolve sample in 125 μL methanol, then dilute to 0.5 mL with distilled water and mix to suspend solid materials.

c. Prewash a C-18 Sep-Pak with 4 mL methanol followed by 4 mL of 10% methanol/water.

d. Using a disposable pipet, transfer the sample to the Sep-Pak, allowing all the sample to drain onto the adsorbent bed. Dilute eluate.

e. Wash tube with two 0.5 mL portions of 10% methanol/water, transferring each to the Sep-Pak as in step d.

f. Slowly (2-3 mL/min) wash the Sep-Pak with an additional 5 mL of 10% methanol/water. Allow to drain completely. Discard the eluate.

g. Place a 50-100 mL round-bottom flask under the Sep-Pak and elute with 6 mL 70% methanol/water at the same rate.

h. Add 3 mL 5% sodium sulfate solution to round-bottom flask, attach to a rotary evaporator with the water bath set to 35° C, and concentrate to 3-4 mL.

i. Using a disposable pipet, transfer the concentrate to a 15 mL centrifuge tube. Rinse flask with 1-2 mL water, adding the rinse to the tube. Final volume should be about 5 mL.

j. Rinse flask with 3 mL dichloromethane (DCM), transferring rinse to the centrifuge tube containing the aqueous extracts.

k. Stopper tube and extract the aqueous concentrate by shaking vigorously or mixing on a vortex mixer for 30 sec.

l. Using a Pasteur pipet, transfer lower phase onto a 0.5 × 2.5 cm sodium sulfate column prepared in a disposable pipet, which has been prewashed with 4-5 mL DCM. Drain the column into a second 15 mL centrifuge tube.

m. Repeat steps k-l twice, using 2 mL DCM for the final two extractions.

n. Concentrate solution just to dryness using an N-Evap with a bath temperature of 35° C.

o. Redissolve residue in 0.25 mL ethyl acetate. Mix by vortexing for 10 sec.

p. Using a disposable pipet, transfer the sample to a 1 mL reaction vial.

IIA. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP (Continued)

2. Derivatization

a. Add 25 μL of heptafluorobutyric anhydride (HFBA) to the ethyl acetate solution in the vial, cap tightly, and heat for two hours at 60° C.

b. Allow the mixture to cool; then transfer it to a 15 mL centrifuge tube containing 6 mL 0.1M phosphate buffer using a Pasteur pipet.

c. Rinse the reaction vial five times with 1 mL aliquots of pentane, and transfer the rinses to the centrifuge tube. Stopper tube and shake for 30 sec to hydrolyze residual HFBA.

d. Transfer upper pentane layer to another centrifuge tube, taking care not to transfer any aqueous phase.

e. Concentrate the pentane extract just to dryness on an N-Evap, using a water bath temperature of 30-35° C. Dilute to an appropriate volume with hexane and mix thoroughly. Adjust the volume so that analyte concentration is sufficient to produce a signal to noise ratio of at least 3-4 for the least intense ion peak monitored. This concentration must be determined experimentally prior to the actual run using derivatized standards. A good initial value is 1 μ/mL.

NOTE: HFBA derivatives are susceptible to hydrolysis. Contact time with the buffer solution used to hydrolyze excess HFBA should be kept as short as possible. If possible, analyze derivatives the same day they are made. If derivatized samples cannot be analyzed immediately, they should be stored in a freezer.

IIA. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions and parameters.

Injection mode: Grob splitless.

Injection volume: 1 μL.

Injector temperature: 170° C.

Interface temperature: 270° C.

GC oven temperature program: Hold 60° 1 min. Ramp to 190° Cat 15°/min; then ramp to 265° C at 10°/min. Hold at 265° C for 7 min.

Carrier gas: Helium at 2.5 psi head pressure.

Injector purge delay: 0.6 min.

b. MS conditions and parameters.

Electron multiplier voltage: Autotune voltage or higher.

Start time: 1.5 min prior to elution of sample peak.

Stop time: 1 min after elution of sample peak.

SIM dwell time: 40 milliseconds/ion.

NOTE: The GC/MS system should be tuned just prior to starting a confirmation series. Use of a low resolution mode is acceptable.

NOTE: Operating parameters specified are intended as guidelines and may be adjusted to compensate for variations in equipment used. GC elution parameters should be adjusted so that all compounds of interest can be resolved with good peak shapes. Adjust MS parameters for best instrument performance.

2. Required Samples

A tissue blank and a recovery (fortified blank) containing the compound(s) of interest must accompany the confirmation sample. In addition, a separate external standard containing the surrogate and compound(s) of interest must be derivatized (refer to section E.2) along with the three tissue extracts. Inject 1 μL aliquots of the external standard, confirmation sample, recovery, and blank into the GC/MS as a confirmation set.

IIA. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

a. Except for bufencarb, the following criteria are required for confirmation of carbanate residues:

i. Retention time specification.

Retention times of sample peak(s) should match those of standard or recovery within 1%.

ii. Specific ions required (refer to Table 1 on the following page):

(1) At least 4 of the 6 ions monitored should be present unambiguously.

(2) Ratios of at least 2 ion peaks relative to the base peak as specified in Table 1 should match those of the standard or recovery within 15%.

b. Criteria for confirmation of bufencarb.

When 3 or more component peaks are present in the chromatogram, confirmation may be assumed if the following criteria are met:

i. Retention time specification.

Retention times for at least 3 of the peaks in the sample match those in the standard or recovery within 1%.

ii. Specific ion/ion ratio requirements.

(1) At least 3 of the monitored ions are unambiguously present in at least 3 of the monitored peaks.

(2) Ratios of at least 2 ion peaks relative to the base peak specified in Table 1 should match the standard or recovery within 20% for at least one of the monitored peaks.

IIA. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Table 1. Mass Spectrometric Confirmation Parameters

Compound	*Retention Time*	*Ions Monitored (see note below)*		
Isoprocarb	8.25	226 (.7)	254 (.3)	162 (1.0)
		299 (.2)	317 (.8)	389[m] (.1)
Proxopur	8.95	226 (.5)	254 (.5)	137 (.4)
		306 (1.0)	363 (.3)	405[m] (.2)
Bendiocarb	9.01	226 (.2)	347 (1.0)	348 (.1)
		362 (.1)	404 (.1)	419[m] (.3)
Promecarb	9.28	226 (.06)	254 (.04)	331 (1.0)
		332 (.2)	346 (.5)	403[m] (.2)
Carbofuran	9.61	226 (.4)	254 (.2)	345 (.6)
		360 (1.0)	190 (.4)	417[m] (.7)
3-OHCarbofuran	10.21	226 (.2)	254 (.1)	343 (1.0)
		358 (.3)	359 (.2)	416[a] (.1)
Dioxacarb	10.56	226 (.2)	210 (.4)	149 (1.0)
		317 (.2)	361 (.1)	418[m] (.04)
Carbaryl	10.77	226 (.2)	115 (1.0)	143 (.7)
		340 (1.0)	341 (.2)	397[m] (.6)
Methiocarb	11.32	226 (.04)	167 (.3)	349 (.1)
		364 (1.0)	365 (.1)	421[m] (.4)
Methiocarb[c]	12.4	226 (.4)	254 (.3)	165 (1.0)
		349 (.3)	362 (.3)	420[b] (.1)
Bufencarb A	9.37	226 (.3)	254 (.2)	190 (.5)
		317 (1.0)	374 (.3)	417[m] (.05)
Bufencarb B	9.77	226 (.2)	254 (.3)	303 (1.0)
		331 (.9)	360 (.6)	417[m] (.2)
Bufencarb C	9.91	226 (.1)	254 (.05)	317 (1.0)
		360 (.2)	374 (.3)	417[m] (.2)
Bufencarb D	10.29	226 (.1)	304 (.2)	303 (1.0)
		331 (.8)	388 (.3)	417[m] (.1)
Bufencarb E	10.44	226 (.04)	317 (1.0)	318 (0.1)
		360 (.06)	374 (.1)	417[m] (.07)

NOTE: Values in parentheses are typical ion ratios, normalized to 1.0, and rounded to the nearest tenth (unless <.1)

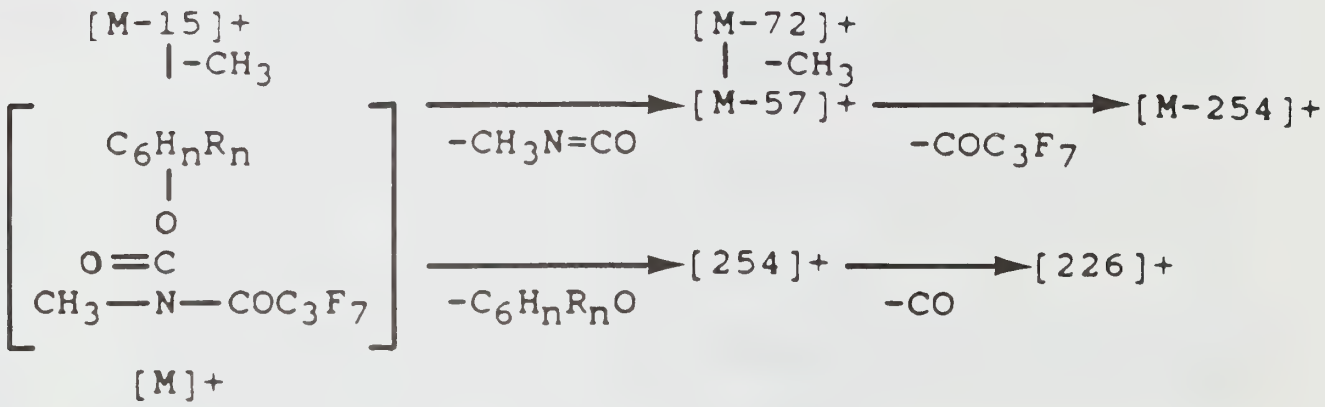
[m] Molecular ion
[c] Double derivative of methiocarb sulfoxide
[a] Molecular ion – OH)
[b] Molecular ion = 633 (abundance too low to use)

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

4. Structures of Confirming Ions

a. HFBA derivatives of carbamate insecticides fragment in the following manner:



where $C_6H_nR_n$ is a substituted phenyl group characteristic of each individual carbamate. Depending on the nature of the substituents on the phenyl group, the ions [M]+, [M-57]+, and [M – 254]+ can undergo additional degradation as those substituents are fragmented. A list of major fragments for each compound is given below:

HFC Derivative	Fragment Ions			
Isoprocarb	[M] 389	[M – 72] 317		
Proxopur	[M] 405	[M – C(CH$_3$)2] 363	[M – 57 – C(CH$_3$)2] 306	[(306) – C$_3$F$_7$] 137
Bendiocarb	[M] 419	[M – 15] 404	[M – 57] 362	[M-72] 347
Promecarb	[M] 403	[M – 57] 346	[M – 72] 331	[M – 72 + H] 332
Carbofuran	[M] 417	[M – 57] 360	[M – 72] 345	
3-OHCarbofuran	[M – OH] 416	[M – 57 – OH] 359		
Dioxacarb	[M] 418	[M – 57] 361	[M – 57 – CH$_2$CH$_2$O] 317	
Carbaryl	[M] 397	[M – 57] 340	[M – 254] 143	[M – 254 – CH$_2$CH$_2$] 115
Methiocarb	[M] 421	[M – 57] 364	[M – 72] 349	[M – 254] 167
Bufencarb (A, C, E)	[M] 417	[M – C$_3$H$_7$] 374	[M – 57] 360	[M – 57 – C$_3$H$_7$] 317
Bufencarb (B, D)	[M] 417	[M – 57] 360	[M – 57 – C$_2$H$_5$] 331	[M – 57 – 2(C$_2$H$_5$)] 303

IIA. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

4. Interferences

There are no known interferences to this procedure. Interferences resulting from co-eluting compounds are a function of individual sample and the efficiency of the cleanup and cannot be predicted.

H. HAZARD ANALYSIS

1. Method Title

Refer to Determinative Method, section H.1.

2. Required Protective Equipment

Refer to Determinative Method, section H.2.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Pentane Methanol HFBA	These compounds are flammable and can produce toxic effects through exposure to skin, eyes, respiratory system, or ingestion.	All procedures involving volatile reagents should be carried out in an efficient fume hood away from any source of ignition. Protective gear should worn when the possibility of solvent spillage exists.

4. Disposal Procedures

Reagent mixtures used in section E.	See above.	Hold in designated storage cans awaiting disposal by the contractor or in-house specialist.
Sep-Pak columns saturated with solvent.	See above.	Allow columns to dry in a fume hood. After dried, may be discarded in regular trash container.

IIA. CONFIRMATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Confirmation of Carbamates*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

Carbamate Confirmation Worksheet

Analyst: _____

Date Started: _____

Date Completed: _____

Sample	Ret. Time	Ion Monitored(1)	Abundance(2)	Ion Ratio(3)
		Ion 1:		
		Ion 2:		
		Ion 3:		
		Ion 4:		
		Ion 5:		
		Ion 6:		
		Ion 1:		
		Ion 2:		
		Ion 3:		
		Ion 4:		
		Ion 5:		
		Ion 6:		
		Ion 1:		
		Ion 2:		
		Ion 3:		
		Ion 4:		
		Ion 5:		
		Ion 6:		
		Ion 1:		
		Ion 2:		
		Ion 3:		
		Ion 4:		
		Ion 5:		
		Ion 6:		
		Ion 1:		
		Ion 2:		
		Ion 3:		
		Ion 4:		
		Ion 5:		
		Ion 6:		
		Ion 1;		
		Ion 2:		
		Ion 3:		
		Ion 4:		
		Ion 5:		
		Ion 6:		

1. Refer to Table 1. in confirmatory method for ions to monitor.
2. Enter peak height or area calculated from ion chromatogram
3. Ion ratio calculated relative to most abundant ion

IIA. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positive at 0 ppb for all compounds.

b. No false negatives at 10 ppb for all compounds.

2. Critical Control Points and Specifications

The following section refers only to the derivatization process of the confirmatory method. Initial sample clean up and preparation follow the same criteria as in Determinative Method.

Record	*Acceptable Control*
Evaporation temperature	< 35° C ± 5° C
Heptafluorobutyric anhydride:	
Volume added	25 μL ± 2μL
Heating temperature	60° C ± 5° C
Heating time	2 hr ± 10 min
Final Sample volume	Varies depending on analyte concentration

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Inject derivatized standards and determine the relative ratios of the ions of interest for each component peak of all compounds to be studied. Determine instrument sensitivity for each compound of interest.

ii. Phase II: For each species to be monitored, clean up, derivatize, and inject:

(a) A tissue blank.

(b) Recoveries of all compounds of interest at least once at their specified confirmatory levels (more than one compound may be included in a recovery).

iii. Phase III: Check samples for analyst accreditation.

(a) Analyze a total of 3 recovery sets on 3 separate days for each species/analyte pair.

(b) Report data to Chemistry Division, QSB.

b. Acceptability criteria.

Analyst must demonstrate the ability to meet confirmation criteria in section F. 3 without any false positives.

IIA. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples	a. System—minimum contents. i. Frequency—At least one check sample semiannually per analyst. ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer. b. Acceptability criteria. Ability to confirm samples using the criteria in section F. 3 without any false positives.
5. Sample Acceptablility and Stability	Refer to Determinative Method, section J.5.
6. Sample Set	a. The presumptive suspect sample. b. A blank tissue fortified at the level found in that sample. c. A tissue blank.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): 10 ppb.

IIB. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory

This method is used to confirm residues of aldicarb and aldicarb sulfoxide.

Residues of aldicarb, aldicarb sulfoxide, and aldicarb sulfone in the extract obtained from the Determinative Method are separated, if necessary, using normal phase SPE. Aldicarb and aldicarb sulfoxide are oxidized to their corresponding sulfones using peracetic acid, and reanalyzed by liquid chromatography. The disappearance of aldicarb or aldicarb sulfoxide combined with the appearance of its oxidation product provides confirmatory evidence of the compound's presence.

2. Applicability

This method is applicable to all tissues referenced in the Determinative Method, section A.2.

B. EQUIPMENT

1. Apparatus

NOTE: Refer to Determinative Method, section B.1. Equivalent equipment may be substituted unless noted.

a. Centrifuge tubes: Graduated 15 mL tubes with glass stoppers. Pyrex #8084.

b. Disposable pipet, Pasteur-type: Kimble #72050.

c. Vortex mixer: Thermolyne Maxi-Mix.

d. Evaporative concentrator: Meyer N-Evap model 112 using nitrogen purge gas, with water bath.

e. Sep-Pak silica cartridges: Waters Assoc., Milford, MA. Verify elution parameters for every lot used.

2. Instrumentation

Refer to Determinative Method, section B. 2.

IIB. CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Peracetic acid, 35% solution: FMC Corp., Buffalo, NY. Store in refrigerator.

b. Sodium bicarbonate, reagent grade: Mallinckrodt.

c. Sodium sulfate, anhydrous, reagent grade: Mallinckrodt.

d. Solvents: All solvents should be pesticide quality (Burdick and Jackson, distilled in glass or equivalent.

   i. Acetone.

   ii. Dichloromethane.

   iii. Methanol.

   iv. Chloroform.

   v. Acetonitrile.

e. Peracetic acid, 1.75%: Dilute 5.0 mL 35% peracetic acid to 100 mL with acetone. Store in refrigerator. Stable for at least a month.

f. Buffer solution: Prepare 0.25N sodium bicarbonate solution by dissolving 105 g in 500 mL distilled water.

D. STANDARDS

Refer to Determinative Method, section D.

IIB. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP

1. Extraction

a. Extract and clean up samples as described in Determinative Method, section E.1, steps a through t. Ignore step u and transfer the entire sample onto the Bond-Elut cartridge in step v. Continue with procedure through step x. At this point, split the sample into two equal parts and evaporate both tubes to dryness as described in step y.

b. Mark one of the tubes ''control.'' Add 200 υL of methanol to this tube and vortex for 5 sec. Stopper tube and save for use in section F.1.a below.

c. If the sample contains aldicarb sulfone, it will be necessary to separate aldicarb and aldicarb sulfoxide from the sulfone before proceeding with oxidation steps—proceed with steps i-v immediately below. Otherwise proceed to section E.2.a below.

i. Redissolve the dried extract in the sample tube from step b above in 2 mL of chloroform. Vortex for 10 sec.

ii. Transfer the chloroform extract to a 10 mL syringe surmounting a silica Sep-Pak cartridge. Rinse tube with 8 mL chloroform, and add to syringe. Allow all to drain into a 15 mL centrifuge tube. Add another 5 mL chloroform to the tube and allow to drain into the centrifuge tube. This fraction contains aldicarb, if present. Discard if no aldicarb is present in the sample. If no aldicarb sulfoxide is present in the sample, go to step v below.

iii. Elute a second fraction with 4 mL acetonitrile/chloroform (1 + 1) into a second centrifuge tube. This fraction contains aldicarb sulfone.

iv. Elute a third fraction using 5 mL methanol/chloroform (1 + 1) into a third centrifuge tube. This fraction contains aldicarb sulfoxide, if present.

v. Concentrate the fraction containing the compound of interest just to dryness on an N-Evap using a nitrogen purge and 30 – 35° C water bath. If both compounds are present, concentrate the two fractions to about 2 mL and recombine, taking the mixture to dryness as described above.

2. Oxidation

a. Add 0.3 mL of 5% peracetic acid solution to the sample tube, stopper tightly, and vortex for 5 sec. Allow the mixture to stand at room temperature (≥20° C) for 45 min.

b. Place the tube on N-Evap with a 30° C water bath and remove all traces of acetone using a nitrogen purge. A residue of acetic acid will remain.

c. Slowly add 1 mL of sodium bicarbonate buffer, and mix gently by flicking bottom of tube. Allow the mixture to stand for 10-15 min.

d. Prepare a drying column by plugging the small end of a disposable pipet with glass wool, then adding a 2 cm layer of sodium sulfate. Rinse the column with 3-4 mL dichloromethane.

IIB. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP (Continued)

e. Add 1 mL of dichloromethane to the tube containing the buffer mixture and extract for 10 sec using a vortex mixer.

f. Using a disposable pipet, transfer the lower organic phase from the centrifuge tube onto the drying column, collecting the eluate in another 15 mL centrifuge tube.

g. Repeat steps e and f twice more, pooling all three fractions. Rinse drying column with 0.5-1.0 mL dichloromethane and add to combine eluates.

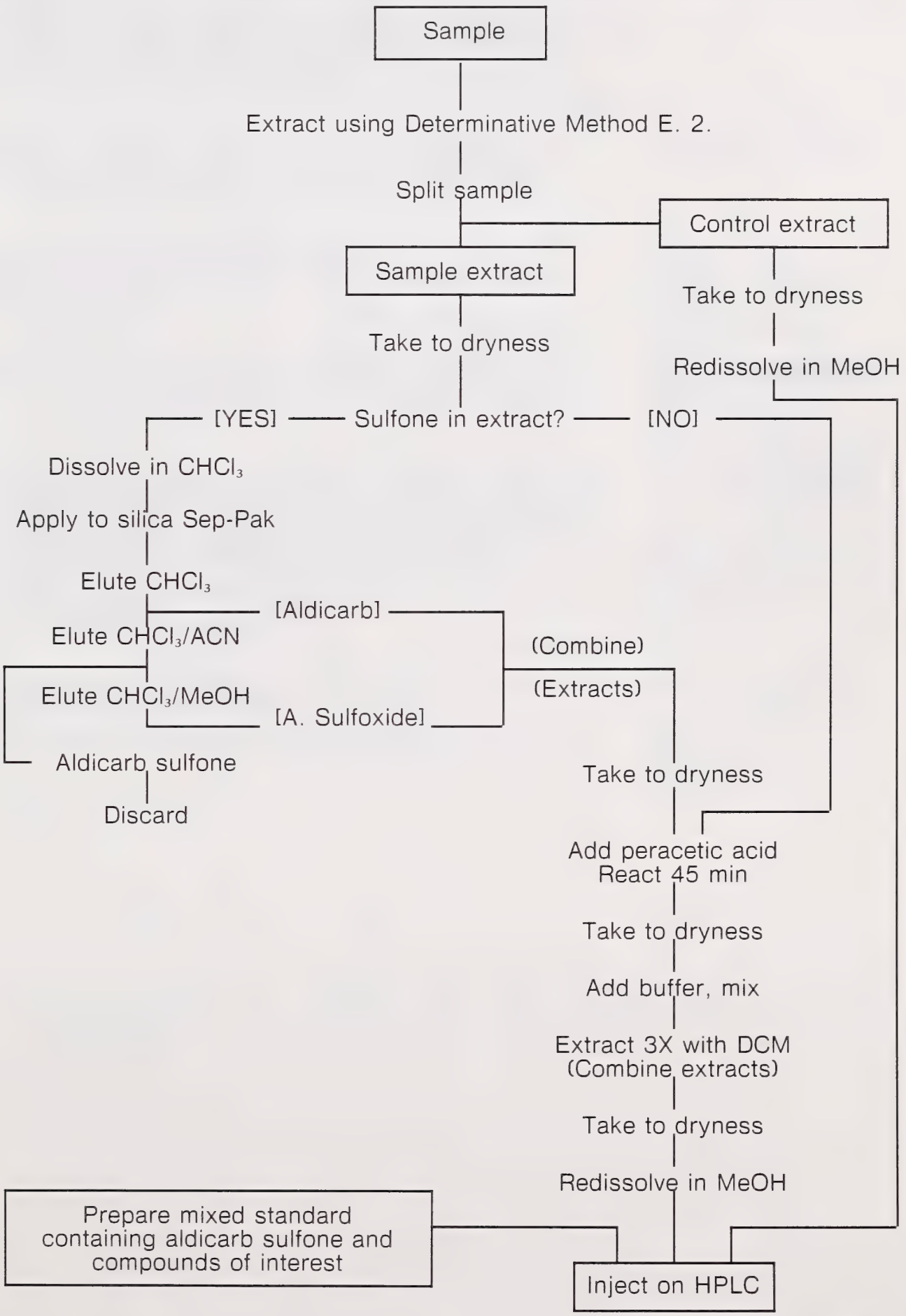
h. Concentrate the solution just to dryness using dry nitrogen on an N-Evap with a 30° C water bath. Add 0.2 mL methanol to dissolve the residue. Stopper and vortex for 5 seconds.

IIB. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP (Continued)

3. Flow Chart Summary

Confirmation of Aldicarb and Aldicarb Sulfoxide



IIB. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. Using the operating conditions described in Determinative Method, section F.1, inject a standard containing aldicarb sulfone and the compound(s) of interest at concentrations approximately equal to those expected to be found in the sample, followed by the control extract from section E.1.b and the oxidized sample extract from section E.2.h.

b. By an acceptable means, measure the peak areas or peak heights for each component in the standard, control, and sample. Calculate the concentrations of aldicarb, aldicarb sulfoxide, and aldicarb sulfone in the control and sample using the formula:

$$\text{ppb compound} = \frac{\text{pk area (sample)} \times \text{conc. (std) [ng/mL]}}{\text{pk area (std)} \times 35 \text{ g/mL}}$$

2. Required Samples

A confirmation set should include the presumptive sample from the determinative method, a tissue blank, and a recovery spiked with the compound(s) of interest at levels similar to those found in the sample.

3. Criteria for Confirmation

A sample can be considered confirmed if there is evidence of both the disappearance of the aldicarb and/or aldicarb sulfoxide, and the appearance of aldicarb sulfone. The amount of sulfone recovered must be within ± 30% of the expected amount. Calculate the difference using the formula:

$$\%\ \text{Difference} = \frac{\text{ppb sulfone found} - \text{ppb sulfone expected}}{\text{ppb sulfone expected}} \times 100\%$$

where:

$$\text{ppb sulfone expected} = \text{ppb (precursor)} \times \text{conversion factor (precursor)}$$

Conversion factors for oxidation products of aldicarb and aldicarb sulfoxide are 1.17 and 1.08 respectively.

IIB. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Method Title

See Determinative Method, section H. 1.

2. Required Protective Equipment

See Determinative Method, section H. 2.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Peracetic acid solutions Acetone Acetonitrile Chloroform Methanol	These compounds are flammable and can produce toxic effects through exposure to skin, eyes, respiratory system, or ingestion.	All procedures involving volatile reagents should be carried out in an efficient fume hood away from any source of ignition. Protective gear should worn when the possibility of solvent spillage exists.

4. Disposal Procedures

Reagent mixtures used in section E.	See above.	Hold in designated storage cans awaiting disposal by the contractor or in-house specialist.
Sep-Pak columns saturated with solvent.	See above.	Allow columns to dry in a fume hood. After dried, may be discarded in regular trash container.

I. WORKSHEET

The worksheet on the following page, *Data Collection Worksheet*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

Data Collection Worksheet
Confirmation of Aldicarb and/or Aldicarb Sulfoxide

Date	Sample No.	Analyst:	
Peak Ht/Area			
Substance Injected	**Aldicarb**	**Aldicarb Sulfoxide**	**Aldicarb Sulfone**
Standard Level =			
Recovery (control) (oxidized)			
Sample (control) (oxidized)			
Blank (control) (oxidized)			

Calculated Concentrations, ppb

Substance Injected	Aldicarb	Aldicarb Sulfoxide	Aldicarb Sulfone
Recovery (control) Level = (oxidized)			
Sample (control) (oxidized)			

Quantitative Results

Substance Injected	Sulfone Found	Sulfone Expected	Difference
Sample			
Recovery			

IIB. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positive at 0 ppb for all compounds.

b. No false negatives at 10 ppb for all compounds.

2. Critical Control Points and Specifications

The following section refers only to the column clean up and oxidation process of the confirmatory method. Initial sample clean up and preparation follow the same criteria as in Determinative Method, section J.2.

Record	*Acceptable Control*
Evaporation (section E.1.a; 1.c.v; and 2.h)	Evaporate just to dryness—do not overdry.
Elution rates (section E.1.c)	Elute at 2-4 mL/min. Use gravity flow if possible.
Oxidation time (section E.2.a)	45-60 min
Oxidation temperature	20-30° C
Methanol volume	200 μL ± 2 μL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Separate mixed standards using column chromatography to verify column performance. Oxidize and inject standard fractions and determine overall efficiency of the procedure.

ii. Phase II: For each species to be monitored, oxidize and inject:

(a) A tissue blank.

(b) Recoveries of all compounds of interest at their specified confirmatory levels. Both compounds may be combined in a single recovery.

iii. Phase III: Check samples for analyst accreditation.

(a) Analyze a total of 3 recovery sets on 3 separate days for each species/analyte pair.

(b) Report data to Chemistry Division, QSB.

b. Acceptability criteria.

Analyst must demonstrate the ability to meet confirmation criteria as in section F.3 without any false positives.

IIB. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples	a. System—minimum requirements. i. Frequency—At least one sample semi-annually per analyst. ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer. b. Acceptability criteria. Refer to section F.3.
5. Sample Acceptability and Stability	Refer to Determinative Method, section J.5.
6. Sample Set	Each sample set would include the presumptive suspect sample, a blank tissue fortified at the level found in that sample, and a tissue blank.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): 10 ppb.

CARBADOX METABOLITE IN SWINE TISSUE

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 3
C. Reagents and Solutions 5
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 12
G. Calculations 17
H. Hazard Analysis 18
I. Worksheet 19
J. Quality Assurance Plan 23

II. Confirmatory Method

A. Introduction 25
B. Equipment 25
C. Reagents 25
D. Standards 25
E. Sample Preparation 26
F. Analytical Confirmation Procedure 26
G. [Reserved]
H. Hazard Analysis 31
I. [Reserved]
J. Quality Assurance Plan 31

I. DETERMINATIVE METHOD

A. INTRODUCTION

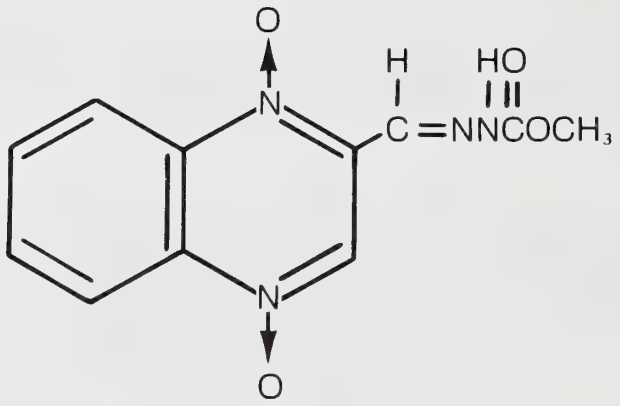
1. Theory and Structures

Carbadox is approved for use in swine weighing less than 75 lbs to prevent or treat enteritis and for increased feed efficiency and weight gain. Since the parent compound is a liver carcinogen, carbadox is monitored in domestic hogs, boars, and sows.

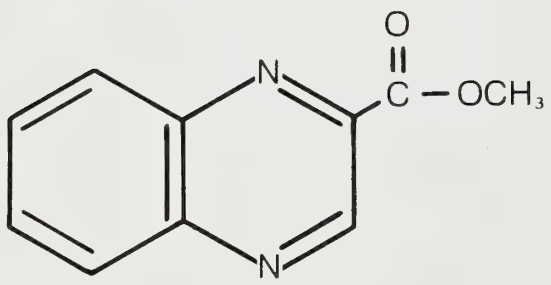
Carbadox metabolic residues are determined as quinoxaline-2-carboxylic acid (QCA), which is isolated from the tissue by alkaline hydrolysis, sequential extraction into ethyl acetate and pH 6 buffer, and ion exclusion chromatography. The column eluate is extracted with chloroform and derivatized with methanolic sulfuric acid. The methyl ester derivative, methyl quinoxaline-2-carboxylate (QME), is then quantitated by GC-ECD and confirmed by GC/MS-SIM.

2. Applicability

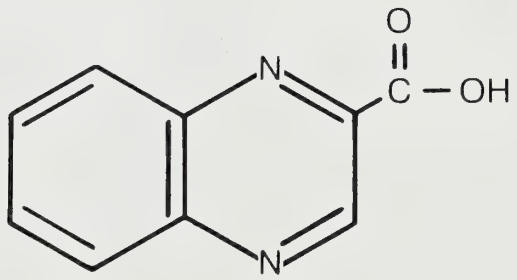
This method has been validated for carbadox analysis in swine liver.



CARBADOX
Methyl 3-(2-quinoxalinyl-methylene) carbazate-N^1,N^4-dioxide



Methyl Quinoxaline-2-carboxylate
"QME" or CP-25,536



Quinoxaline-2-carboxylic Acid
"QCA" or CP-16,505

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted for any apparatus listed below if necessary.

a. GC septum: high-temperature silicone, Teflon-lined (Pierce Chemical Co.).

b. Centrifuge: International size 2, model K.

c. Water bath: temperature adjustment up to 100° C (Thelco, model 83).

d. Pipet, disposable: Pasteur-type.

e. Pipet, automatic transfer: 15 mL and 50 mL.

f. Pipet, measuring: 5 mL.

g. Pipet, volumetric: 1.0 mL and 10.0 mL.

h. Pipet, precision: 0.1 mL and 0.2 mL.

i. Centrifuge tube: heavy duty, 15 mL and 50 mL graduated (60 mL capacity), with glass stoppers (R. C. Ewald, Inc.).

j. Volumetric flask: 1 mL, 100 mL, and 1000 mL capacity, glass stoppered.

k. Chromatography columns: 25 cm × 10.5 mm i.d. with Teflon stopcock and 200 mL reservoir capacity (S.G.A. Scientific, Inc., Cat. #JC-1506).

l. Crystallizing dish: 190 mm (diameter) by 100 mm (height), for oil bath.

m. Round-bottom flask: single neck, 250 mL capacity.

n. Test tube rack, test tube mixer.

o. Separatory funnels: 60 and 250 mL capacity with Teflon stopcocks.

p. Rotary evaporator.

q. Thermo stir hot plate.

r. Magnetic stirring bar.

s. Thermometer: 0°-150° C range.

t. Electro balance: Cahn, model G-2.

u. N-Evap: analytical evaporator using high-purity dry nitrogen for purging (Organomation Associates).

v. Micro Kuderna-Danish evaporative concentrator: 2 mL.

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

2. Instrumentation

Equivalent items can be substituted if necessary.

a. Gas chromatograph: Hewlett Packard 5890 GC.

b. GC column: Pyrex glass, U-shaped, 1.8 m × 4 mm id, packed with 3% silar 10C on Gas Chrom Q, 80-100 mesh (Applied Science, Inc.). Condition the packed column with low gas flow at 250° C for 18 hours.

NOTE: The voltage profile for the detector should plateau at 30 volts or less in the DC mode when a stream of nitrogen gas is passed through the column and the detector.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Chloroform, A.C.S., reagent grade.

b. Methanol, A.C.S., reagent grade.

c. Toluene, distilled in glass grade.

d. Ethyl acetate, distilled in glass grade.

e. Isooctane, distilled in glass grade.

f. Citric acid monohydrate, U.S.P.

g. Sodium hydroxide, pellets, reagent grade.

h. Sodium sulfate, anhydrous, A.C.S., reagent grade.

i. Silicone fluid, SF-81 (50) (General Electric).

j. Hydrochloric acid, A.C.S., reagent grade.

k. Sulfuric acid, A.C.S., reagent grade.

l. Resin, AGMP-50, 100-200 mesh (Bio-Rad Laboratories, No. 1430841).

m. Hydrochloric acid, 1M: Dilute 83.3 mL of concentrated HCl to 1000 mL with distilled water.

n. Methanol:Water (10:90), v/v: Dilute 10.0 mL of reagent grade methanol to 100 mL with distilled water.

o. Methanol:Sulfuric Acid (97:3), v/v: Dilute 3.0 mL of concentrated H_2SO_4 to 100 mL with methanol that has been dried over anhydrous Na_2SO_4. Prepare daily, and use an ice bath to cool the methanol before adding the acid.

p. Sodium hydroxide, 3M and 5M: Dissolve 120 g and 200g, respectively, of NaOH pellets in 1000 mL of D.I. water.

q. Citric acid, 1M: Dissolve 210.0 g of citric acid monohydrate in 1000 mL of distilled water.

r. Citric acid buffer, 0.5M: Adjust the pH of 100 mL of 1M citric acid to pH 6.0 with 5M sodium hydroxide (ca 55 mL) using a previously calibrated pH meter. Adjust the final volume to 200 mL with distilled water. Before making the final pH adjustment, cool the buffer to room temperature.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Quinoxaline-2-carboxylic acid (QCA) and methyl quinoxaline-2-carboxylate (QME) standards can be purchased from:

Pfizer Central Research
Pfizer, Inc., Eastern Point Road
Groton, CT 06340

2. Preparation of Standards

a. Quinoxaline-2-carboxylic acid (QCA) solutions.

i. Stock solution A, 15.0 μg/mL: Dissolve 1.50 mg of quinoxaline-2-carboxylic acid in methanol and dilute to 100 mL with methanol.

ii. Working standard solution B, 0.150 μg/mL: Pipet 1.0 mL of stock solution A into 100 mL volumetric flask and dilute to volume with distilled water.

iii. Working standard solution C, 0.150 μg/mL: Pipet 1.0 mL of stock solution A into 100 mL volumetric flask and dilute to volume with methanol.

iv. GC process standard, 0.015 μg/mL: Pipet 1.0 mL of working standard solution C into a 15 mL centrifuge tube and evaporate to dryness under a stream of nitrogen at 55° C. Add 0.2 mL of methanol:sulfuric acid (97:3), stopper, and incubate at 55° C for 30 minutes. Extract and dilute 1:10 with toluene. Esterify standards concurrently with samples as directed in section E.5 below.

b. Methyl quinoxaline-2-carboxylate (QME) solutions.

i. Stock solution 1, 15.0 μg/mL: Weigh 1.50 mg of methyl quinoxaline-2-carboxylate in 100 mL volumetric flask and dilute to volume with toluene.

ii. GC standard working solution 2, 0.015 μg/mL: Pipet 0.10 mL of stock solution 1 into 100 mL volumetric flask and dilute to volume with toluene.

3. Storage Conditions

Standard solutions can be stored in tightly closed glass bottles at room temperature.

4. Shelf Life Stability

Shelf life stability is dependent upon storage conditions—see section D.3 above.

a. Stock standard—one week.

b. Working standards—prepare fresh as needed.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Liver samples are homogenized using a blender or food processor prior to extraction.

2. Column Preparation

Slurry 7.0 g of AGMP-50, 100-200 mesh resin in 1N HCl and transfer to a 10.5 mm i.d. glass column containing a small glass wool plug. Pack the resin to a height of 4 inches (10.1 cm), using a glass rod, and cap the resin bed with a glass wool plug. Maintain the liquid above the resin.

3. Sample Extraction

a. Sample Fortification

Weigh 5.0 ± 0.1 g of blank tissue to a 50 mL centrifuge tube and fortify with 1 mL of QCA working standard solution B. This will yield a 30 ppb fortified sample.

b. Dissolution and hydrolysis

i. Weigh 5.0 ± 0.1 g of freshly sliced frozen tissue in a 50 mL centrifuge tube.

ii. Pipet 10 mL of 3M sodium hydroxide into the tube, stopper lightly, and place it in a preheated 95°-100° C silicone oil bath for 30 minutes. The level of silicone oil in the bath should exceed that of the tissue sample.

iii. Cool the alkaline hydrolysate in an ice bath and acidify to pH ≤ 1 (pH can be measured with pH paper) with 4 mL of conc. HCl.

iv. Add 15 mL of ethyl acetate to the acidified hydrolysate, stopper, and extract by shaking for 20 seconds.

v. Centrifuge the mixture at 1500 rpm for 5 minutes to clarify the ethyl acetate phase. Recover the ethyl acetate phase with a blow-out pipet equipped with a propipet bulb and transfer the extract to a 60 mL separatory funnel with a Teflon stopcock.

vi. Reextract the hydrolysate with two additional 15 mL portions of ethyl acetate and combine the organic extracts. Do not contaminate the ethyl acetate phase with interfacial material during these extractions.

vii. Add 5 mL of 0.5M citric acid buffer (pH = 6.0) to the ethyl acetate extract, shake, and allow the lower phase to clarify (about 10 minutes).

viii. Collect the aqueous phase in a 50 mL glass-stoppered centrifuge tube.

ix. Reextract the ethyl acetate phase with an additional 5 mL of pH 6 buffer. Allow the aqueous phase to clarify. Combine the aqueous extracts in the centrifuge tube.

x. Add 2 mL of conc. HCl. Mix.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Sample Extraction (Continued)

c. Ion exclusion chromatography—sample elution.

i. Transfer the sample to the ion exclusion column prepared in E.2 above. Drain the extract to the top of the resin bed. Wash the tube and resin with 20 mL of 1N HCl. Drain through the column. Rewash the column with an additional 20 mL of 1N HCl. Discard this and previous effluents from the column.

ii. Place a 250 mL separatory funnel under the column and elute the column with 75 mL of methanol:water (10:90). The column may be allowed to run dry in this step. The flow rate of the effluent should be 1.2 mL/min. The resin may be discarded after each assay or it may be regenerated by washing in sequence with methanol, water, and 1N HCl.

d. Concentration of the quinoxaline-2-carboxylic acid eluate.

i. Add 1.0 mL of conc. HCl to the eluate from E.3.c.

ii. Extract with three 50 mL portions of chloroform. Collect the extract in a 250 mL round-bottom flask.

iii. Evaporate to dryness on a rotary evaporator at 45°-50° C.

iv. Transfer the residue to a 15 mL centrifuge tube by washing the flask with three small portions (1.0 mL each) of methanol. Use a disposable Pasteur pipet to transfer the methanolic solvents.

v. Place the tube in a water bath set at 55° C and evaporate the solvent to dryness under a stream of nitrogen. The residue may be stored at room temperature.

e. Esterification of quinoxaline-2-carboxylic acid.

i. Reconstitute the residue with 0.2 mL of freshly prepared methanol:sulfuric acid (97:3).

ii. Stopper and heat at 50°-55° C in a water bath for 30 min.

iii. Remove the tube from the water bath, add 1.0 mL toluene to the tepid esterification solution, and mix thoroughly in a test tube mixer.

iv. Add 1.0 mL water and mix thoroughly. Centrifuge to clarify.

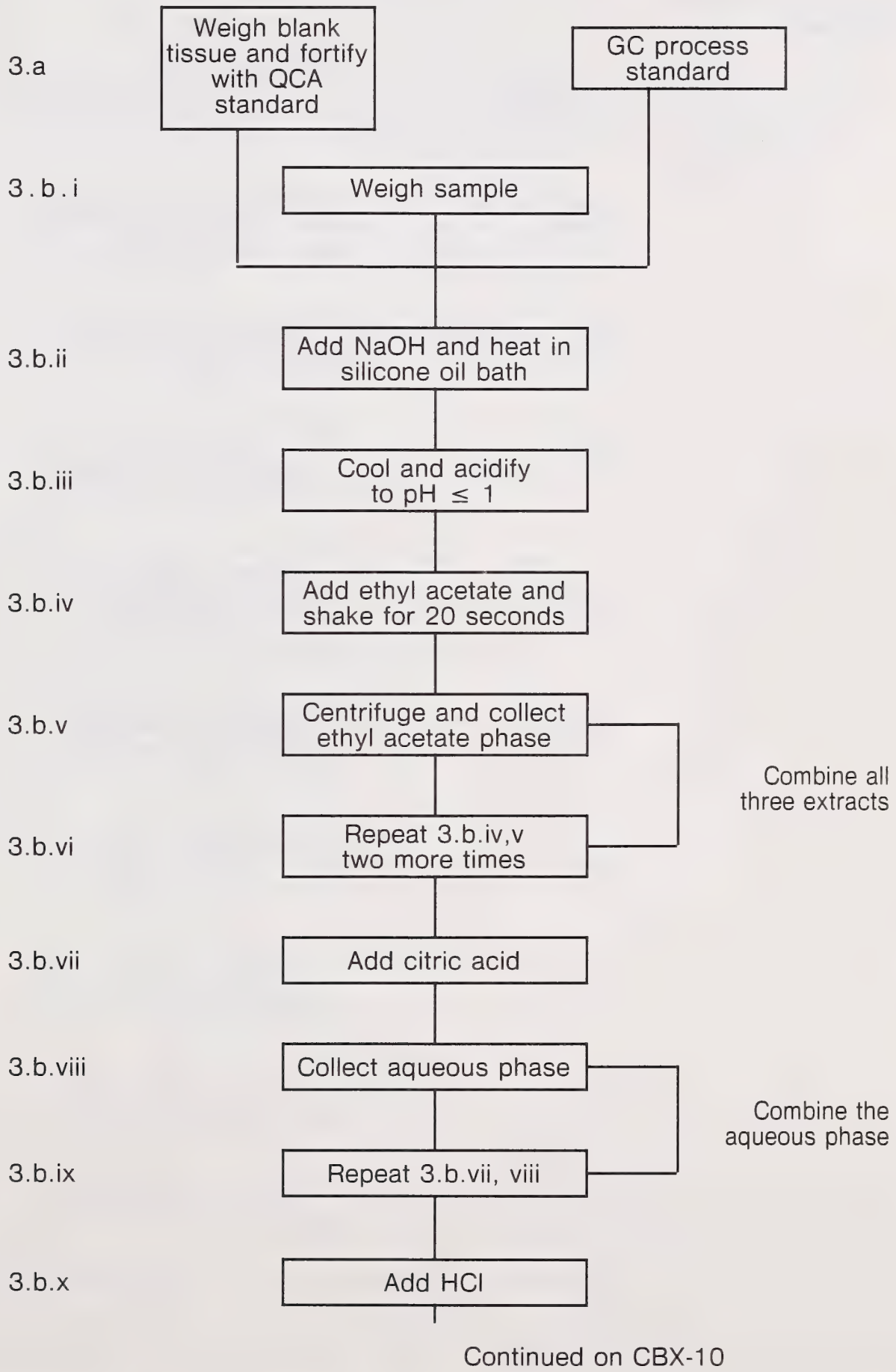
v. Dilute 0.1 mL of the toluene extract to 1.0 mL with toluene. The solution is ready to be quantitated by GC-ECD.

NOTE: The dilutions and biphasic extracts are stable for at least 3 months when stored at reduced temperature to prevent losses through evaporation. The undiluted toluene extract may be utilized for confirmatory identification of QME by GC/MS-SIM.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary

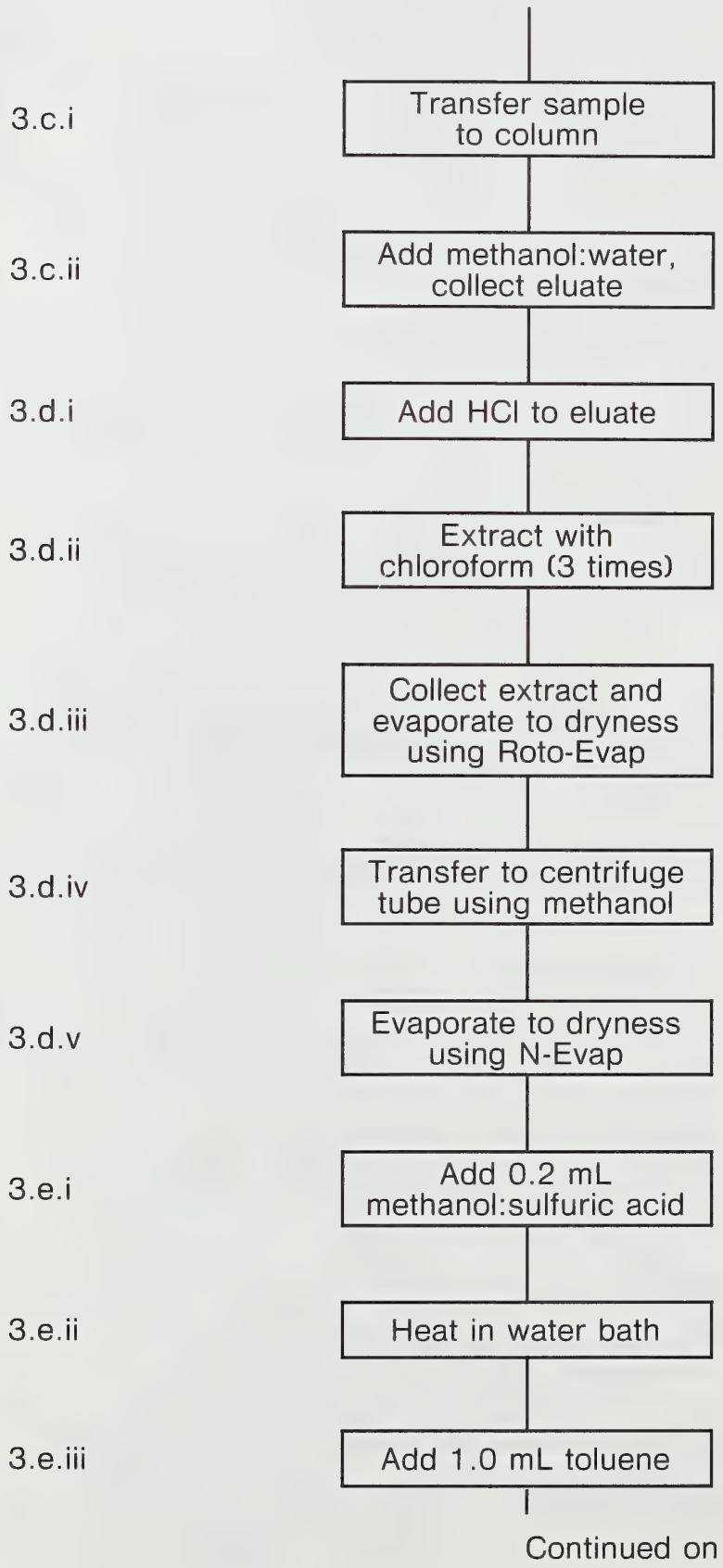


Continued on CBX-10

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary (Continued)

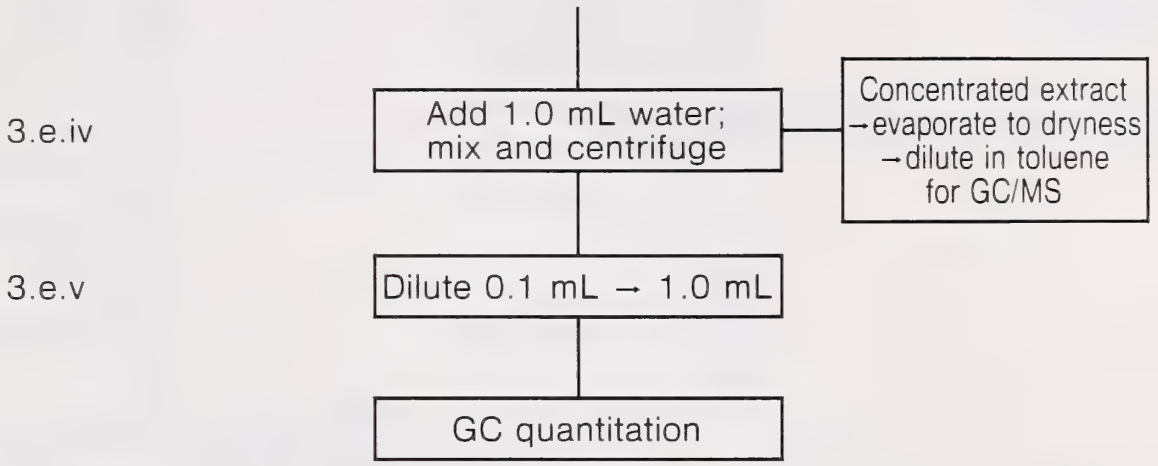


Continued on CBX-11

I. DETERMINATIVE MEHTOD

E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. GC Operating Conditions

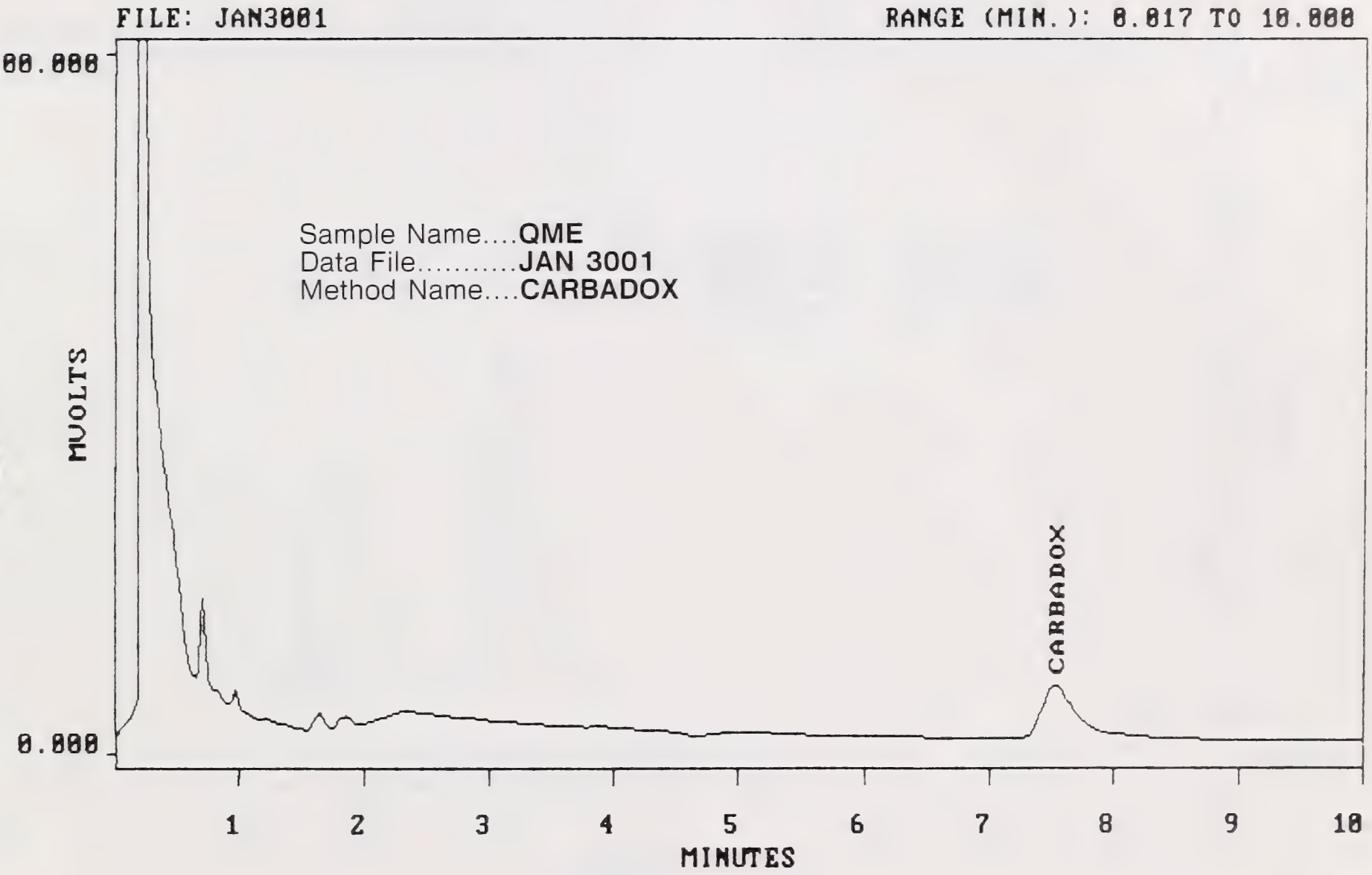
Detector temperature	325° C
Injection temperature	230°-235° C
Oven temperature	
Initial temperature	150° C
Hold time	10 min
Rate	8° C/min
Final temperature	240° C
Carrier gas	Argon-Methane (95:5)
Flow rate	20 mL/min
Injection volume	4 μL
Recorder chart speed	0.5 inches/min
Attenuation	32
Approximate retention time	6.8 min

2. Interferences

None.

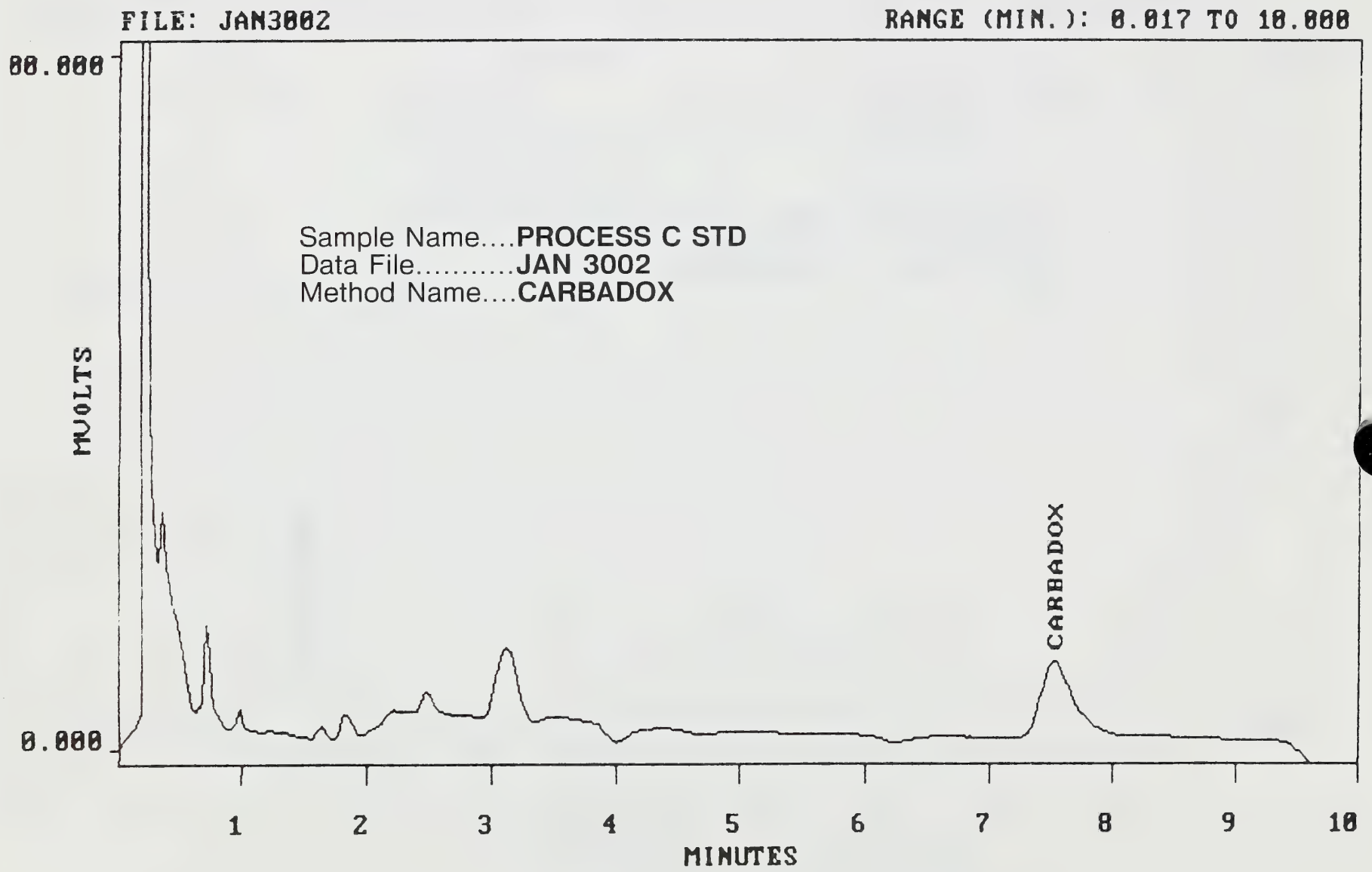
I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



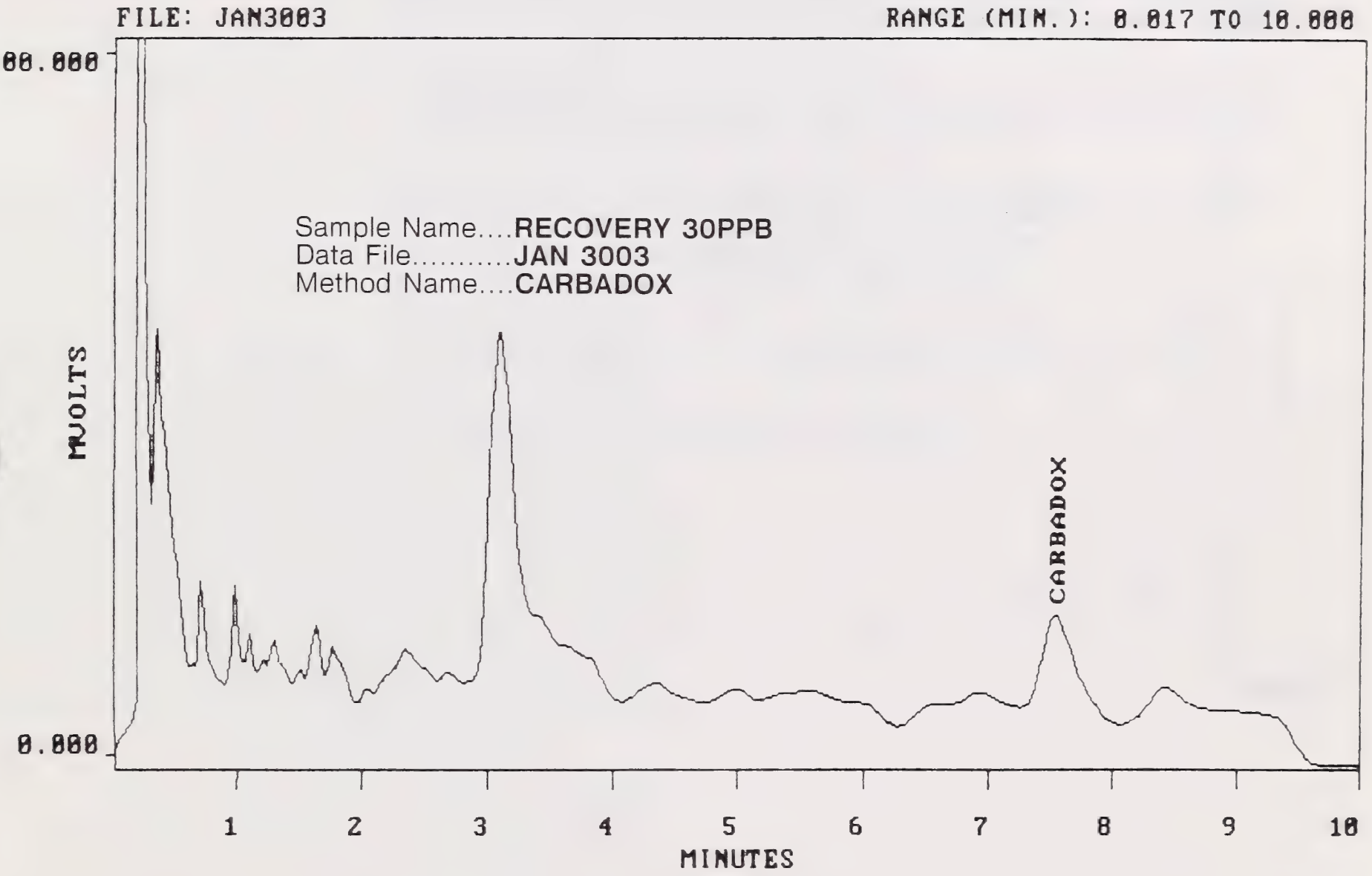
I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



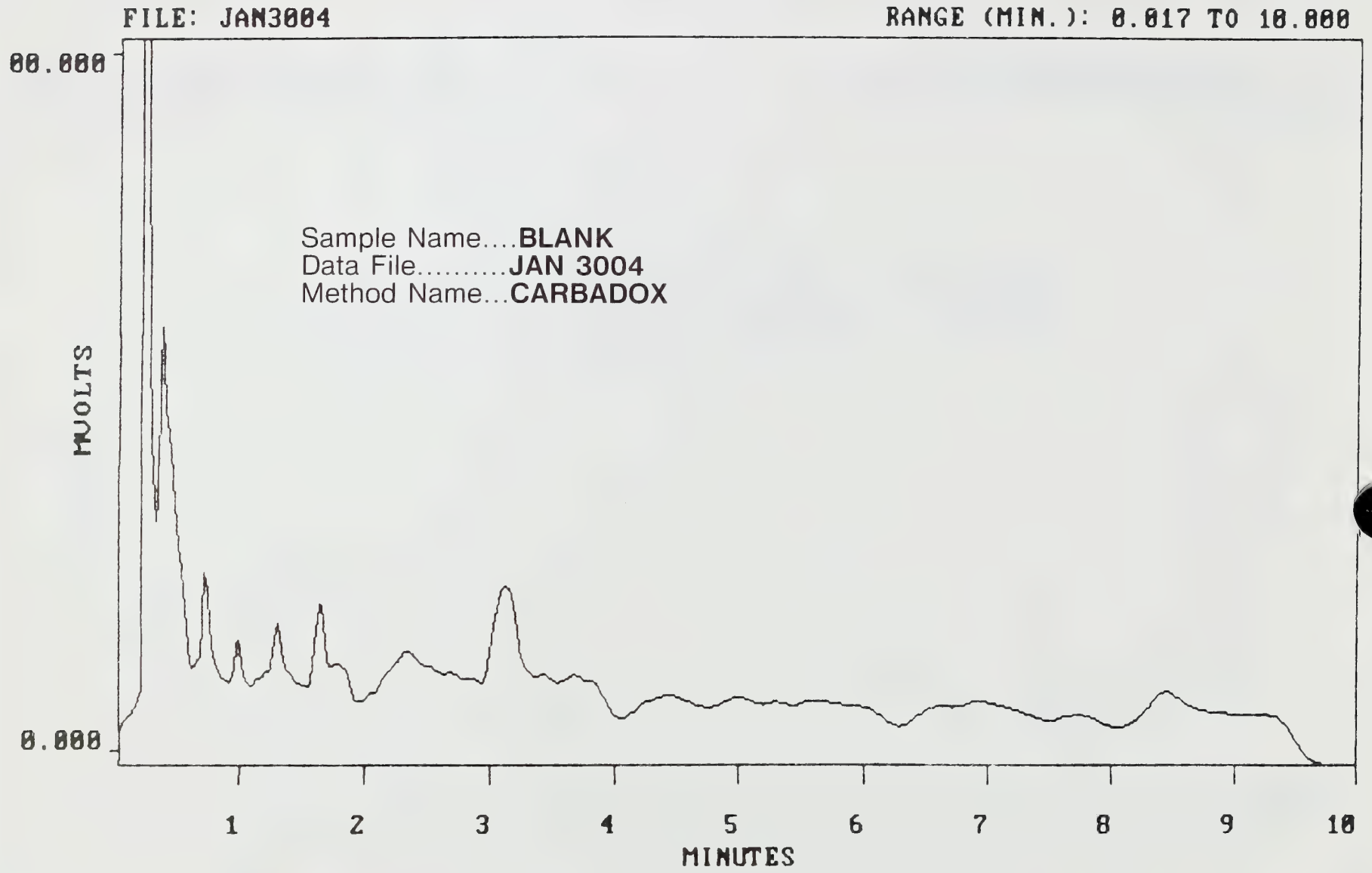
I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Inject 4 μL of reagent blank followed by 4 μL of working standard solution 2 of methyl quinoxaline-2-carboxylate (QME) into the gas chromatograph to determine the retention time of this compound (about 7 minutes) and to evaluate the response of the EC detector. With replicate injections, the peak height of this standard should stabilize at approximately 40% of full scale deflection.

b. Follow QME standard solution 2 with 4 μL of the toluene extract of the process standards and samples. Allow 10 min between sample injections to clear the instrument of background peaks.

c. Measure the peak height (mm) of samples and standards and calculate the results as below:

$$\text{ppb QCA found} = \frac{\text{Peak height of sample} \times 30}{\text{Peak height of process standard}}$$

$$\text{Percent conversion of QCA to QME} = \frac{\text{Peak height of process standard} \times 100}{\text{Peak height of QME} \times 1.08}$$

30 = concentration of process standard (ppb)

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Analysis of Carbadox Metabolite in Swine Tissue

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Chloroform	Listed as a carcinogen by EPA; inhalation of large doses may cause hypotension, respiratory and myocardial depression, and death. Very volatile.	Use under well ventilated hood. Avoid contact with skin, eyes.
Toluene Methanol Ethyl acetate Isooctane	Flammable, poisonous; inhalation will cause headache, fatigue, nausea.	Same as above.
Sodium hydroxide	Very corrosive. Ingestion will result in vomiting, collapse. Will generate heat if solution is made up from NaOH pellets.	Same as above.
Sulfuric acid Hydrochloric acid	Flammable. Skin burns upon contact. Ingestion may cause severe corrosion of mouth and G.I. tract. Eye irritation.	Same as above.
D. Standards		
QCA QME	Limited toxicological data from Pfizer. No evidence of carcinogenicity was found in long-term studies in rats.	Same as above.

4. Disposal Procedures

Organic solvents	See above	Dispose of in proper container and segregate chlorinated from nonchlorinated.
All acid/base solutions	See above	Flush down sink with lots of water. Use separate sink for acid/caustic.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Carbadox*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

CARBADOX

Analyst: _____

Date: _____

Sample Identification	Critical Control Points									Comments
Procedure Key	Sample Weight (g) E.3.a	Oil Bath Temp (C°) E.3.b.ii	Sample pH E.3.b.iii	Resin Weight (g) E.2	Column Flow Rate (mL/min) E.3.c.ii	Roto Vap Temp (C°) E.3.d.iii	Methanol: Sulfuric Acid (mL) E.3.e.i	Extract Diln (mL) E.3.e.v	Results (ppb) G.3	
1. Control										% Rec. = _____
2. Fortified (30 ppb)										10-Av. = _____
3.										
4.										
5.										
6.										
7.										
8.										
9.										
10.										
11.										
12.										

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Analytical Range (ppb)	*Acceptable Recovery %*	*Repeatability %CV_o*
15-60	50-100	20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5.0 ± 0.1 g
b. Silicone oil bath temperature	95-100° C
c. pH adjustment of the alkaline hydroxylate	≤ 1
d. Resin weight	7.0 ± 0.1 g
e. Column effluent flow rate	1.2 mL/min
f. Rotary evaporator temperature	45-50° C
g. N-Evap temperature	≤ 55° C
h. Methanol-sulfuric acid	0.2 mL
i. Extract dilution	0.1 mL → 1.0 mL in toluene

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Triplicate standard curve at each of the following concentration levels: blank, 15 ppb, 30 ppb, and 60 ppb.

ii. Phase II: Fortified samples—Analyses of fortified samples at 0, 15, 30, and 60 ppb on 3 consecutive days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst. At least one out of four of the positive samples is to be confirmed by mass spectrometry.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on difference between replicates.

(c) All % recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Swine liver.

b. Sample receipt size, minimum: 30 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage:

i. Time: 6 months.

ii. Condition: −20° C.

6. Sample Set

Each set must contain a reagent blank, a blank tissue, a blank tissue fortified at 30 ppb, and at the highest concentration found in the suspect sample, and up to 12 additional samples.

7. Sensitivity

a. Lowest detectable level (LDL): 7.5 ppb.

b. Lowest reliable quantitation (LRQ): 30 ppb.

c. Minimum proficiency level (MPL): Not determined.

II. CONFIRMATORY METHOD

A. INTRODUCTION

Refer to Determinative Method, section A.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.1.

2. Instrumentation

GC/MS: Hewlett Packard 5970B MSD/5890 GC equipped with 30 m DB5 column (J & W Scientific, Inc.), 0.25 mm ID, 0.25 micron film thickness, or equivalent.

C. REAGENTS

All reagents as in Determinative Method, section C, in addition to toluene (distilled in glass, pesticide grade).

D. STANDARDS

1. Source

Refer to Determinative Method, section D.1.

2. Preparation of Standards

a. Stock standard, 400 μg/mL: Dissolve 4 mg of stock standard methyl quinoxaline-2-carboxylate in isooctane and dilute to 10 mL volume.

b. Working standard, 4 μg/mL: Add 100 μL of the stock standard to a 10 mL volumetric flask and dilute to volume with isooctane.

3. Storage Conditions

Refer to Determinative Method, section D.3.

4. Shelf Life Stability

Refer to Determinative Method, section D.4.

II. CONFIRMATORY METHOD

E. SAMPLE PREPARATION

Procedure

Refer to Determinative Method, section E.3.e.v: Transfer the remaining 0.9 mL undiluted toluene (sample extract, recovery standard, and method blank) to a 2 mL Kuderna-Danish evaporator and evaporate to near dryness under a stream of nitrogen at room temperature, then reconstitute in 20 μL of isooctane.

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions:

Detector	MS-EI-SIM
Carrier gas	Helium
Flow rate	Approximately 30 cm/sec
Column head pressure	10 psi
Temperature program	
Initial temperature	40° C
Initial hold time	2 min
Program rate	16° C/min
Final temperature	285° C
Final hold time	5 min
Injector temperature	260° C
Injection mode	Grob splitless, 1 min vent delay
Transfer line	290° C

b. Mass spectrometer conditions:

Analyzer parameters	See note below
Electron energy	70 ev
Electron multiplier voltage	200-600 v above the autotune voltage
Dwell time	200 msec per ion

NOTE: The parameters are first set by the autotune program. then the overall sensitivity is maximized by low-mass enhancement using the lens voltage (i.e., ion focus) as well as by resolution adjustment (i.e., amu offset and amu gain). The sensitivity can be considered adequate when an injection of 4 ng of QME gives an S/N ratio of 5 or better for the M/Z ion 188.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

2. Required Samples

a. Inject 1 μL of 4 ppm QME standard to check the instrument performance.

b. Inject 1 μL isooctane to check for carry-over.

c. Inject 1 μL method blank to check for interferences.

d. Inject 1 μL recovery standard.

e. Inject 1 μL isooctane.

f. Inject 1 μL sample.

3. Confirmation Criteria

a. Selective ions monitoring

Compound = QME (methyl quinoxaline-2-carboxylate).

RT(min)	*Ions monitored* (+) base peak (*) molecular ion	*Ion ratios*
13.19	130 (+), 158, 188 (*)	158/130 = 0.30 188/130 = 0.22

158 = M – 30, loss of CH_2O.

130 = M – 58, loss of CH_2CO_2 from molecular ion and loss of CO from m/z 158.

b. Acceptability criteria.

i. All three ions monitored must be present.

ii. The ion ratios, relative to the most intense ion, must be within 15 percent of those found in the recovery sample spiked at the level found in the suspect sample.

iii. The retention time must be within 5 seconds of that found for the recovery sample.

4. Reference

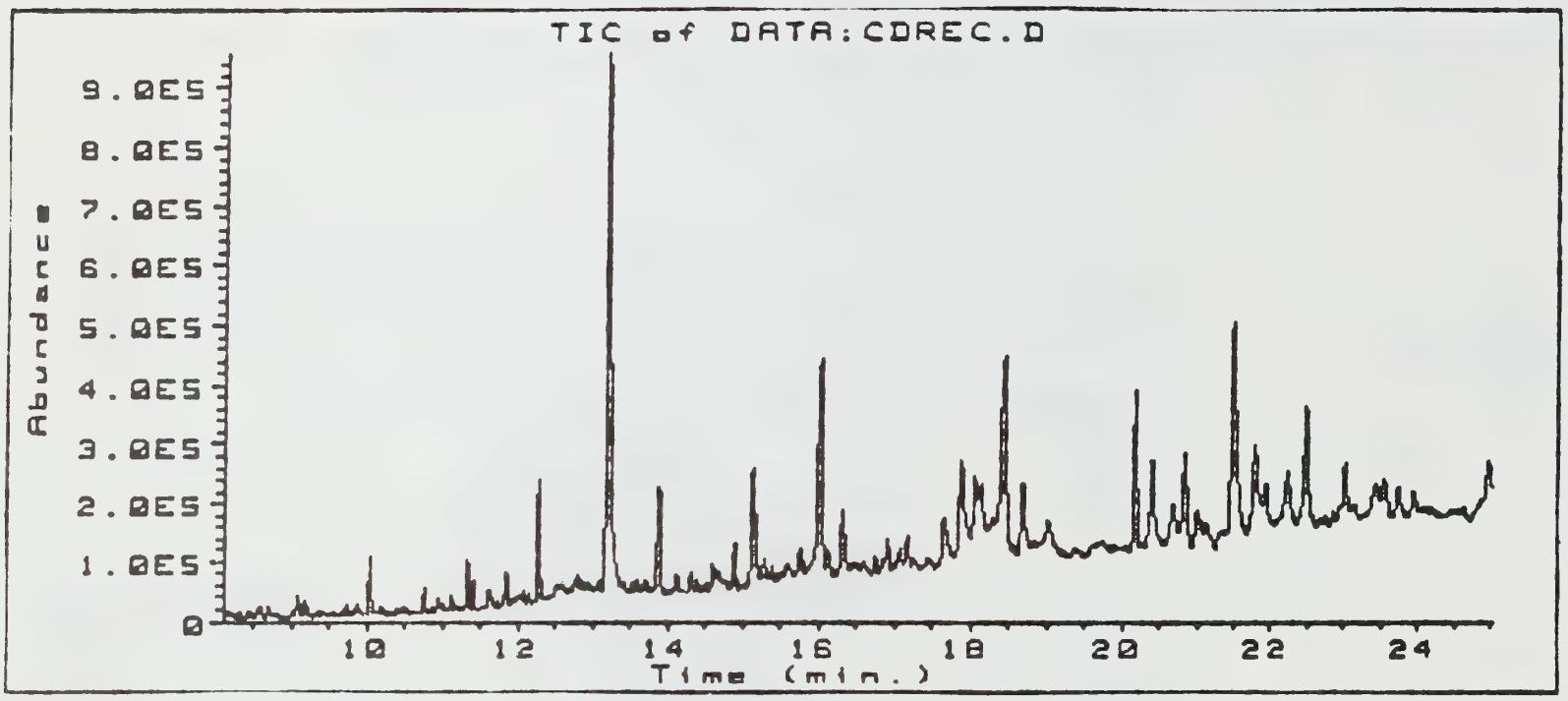
Animal Drug Analytical Manual, U.S. Department of Health and Human Services, Food and Drug Administration. Center for Veterinary Medicine, 1985 Edition.

Contributors: John Wong, Michael Webb.

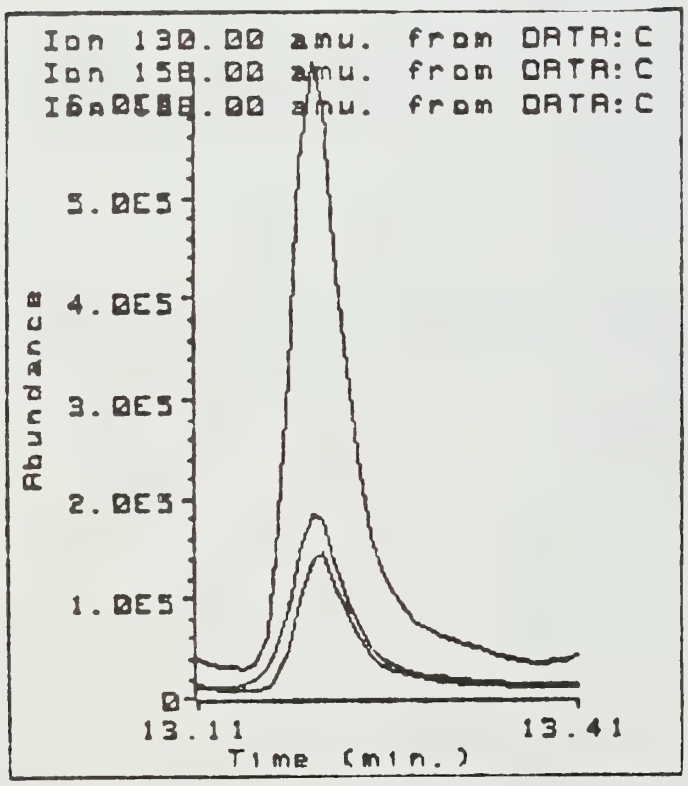
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

TOTAL ION CHROMATOGRAM OF RECOVERY STANDARD



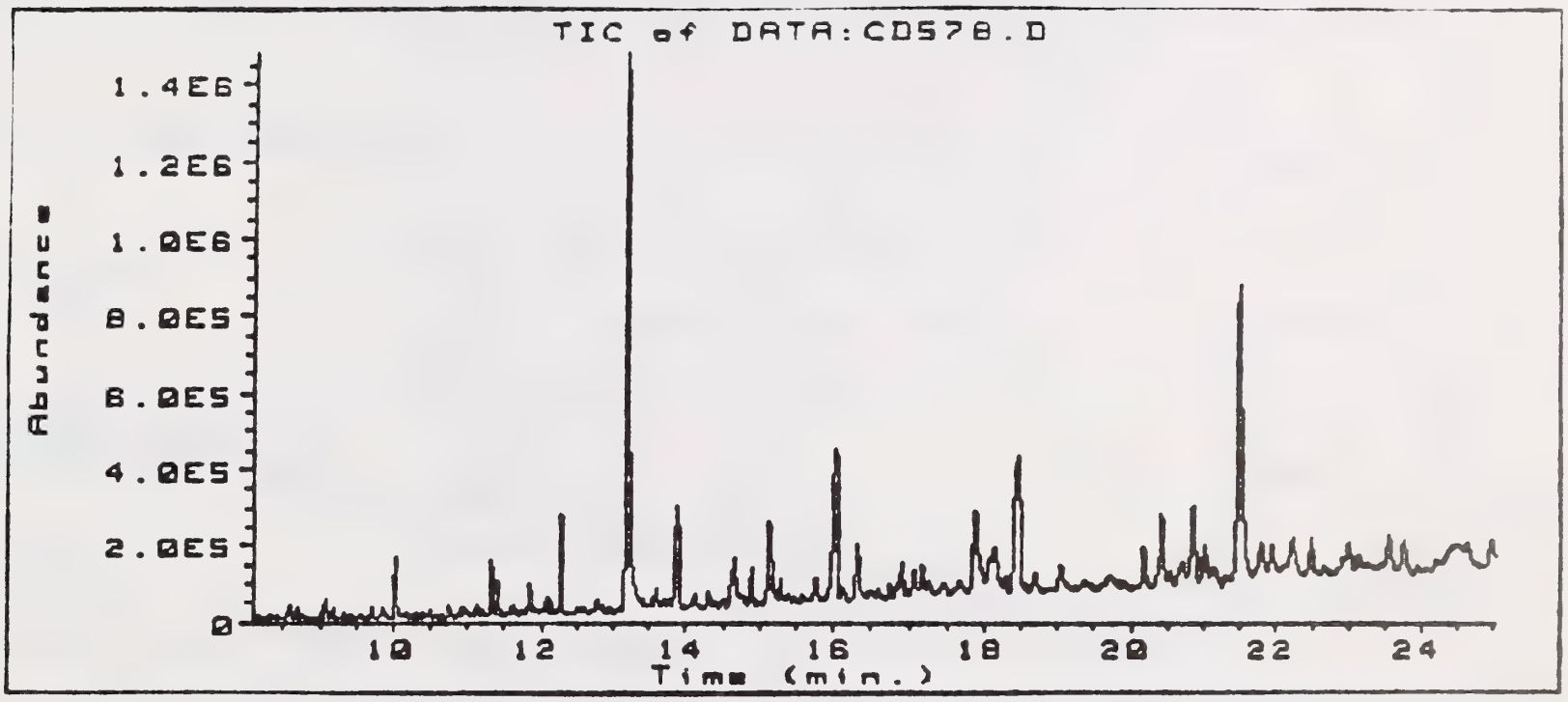
EXTRACTED ION CHROMATOGRAM OF CARBADOX IN RECOVERY STANDARD



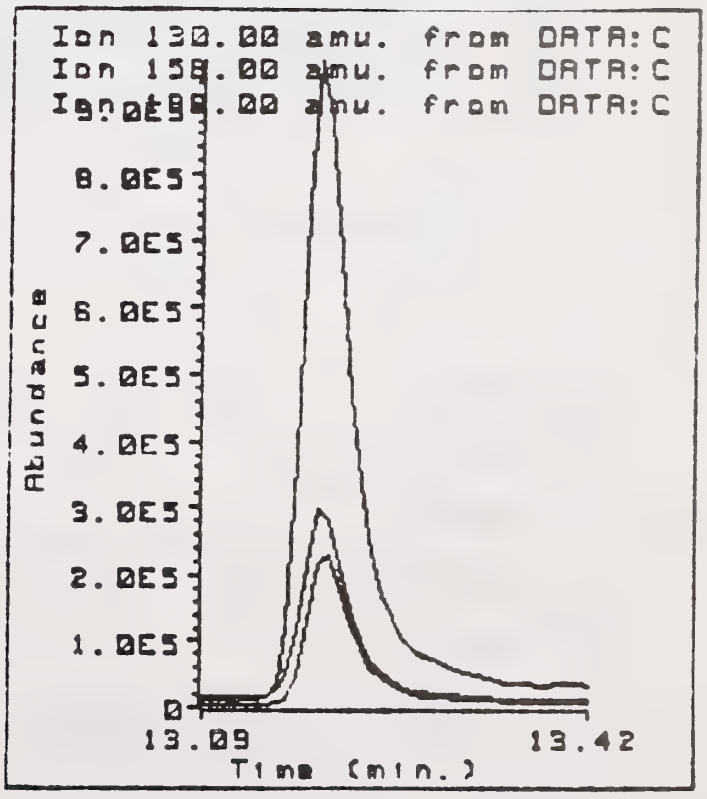
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

TOTAL ION CHROMATOGRAM OF SAMPLE



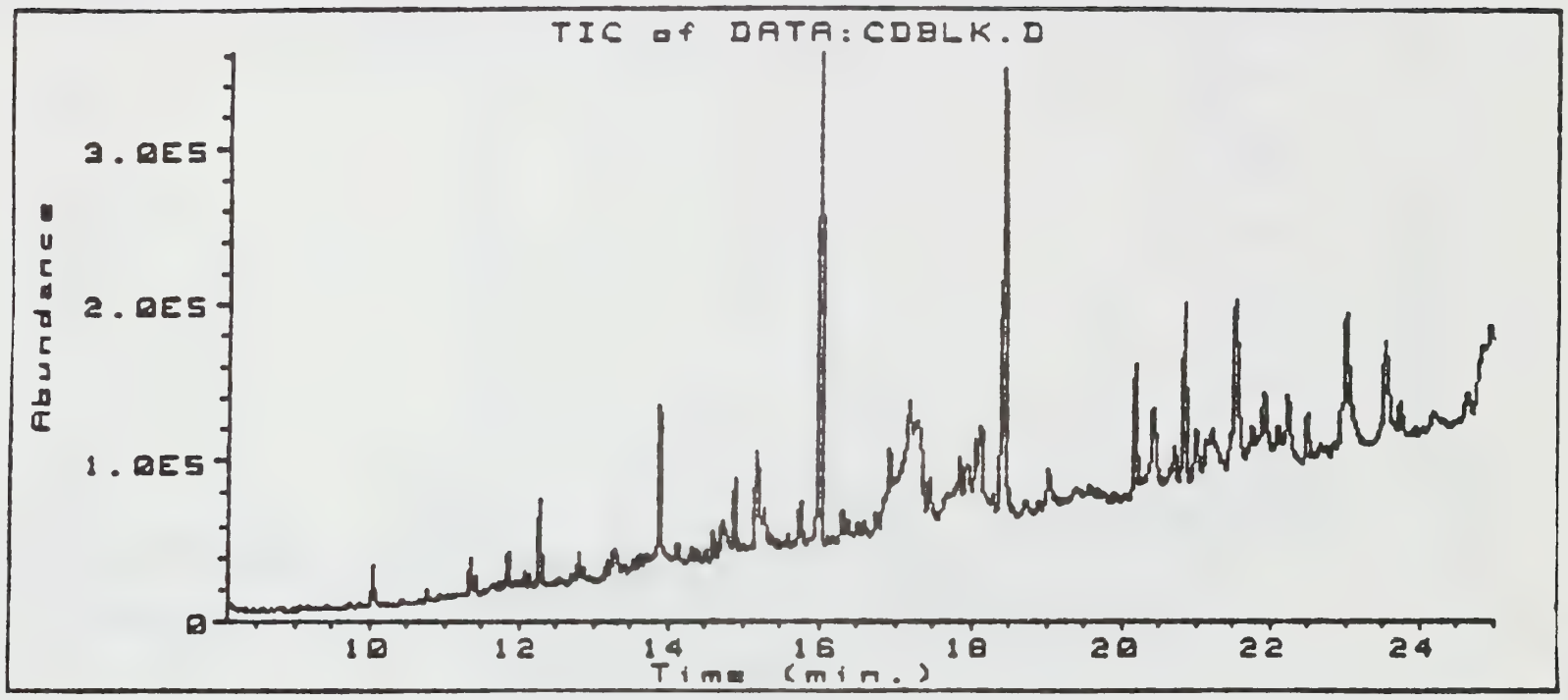
EXTRACTED ION CHROMATOGRAM OF CARBADOX IN SAMPLE



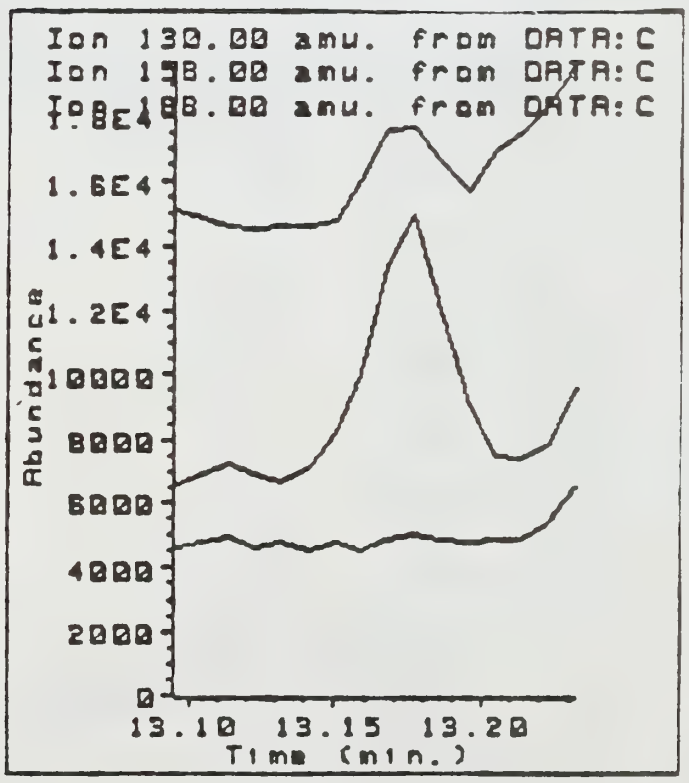
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

TOTAL ION CHROMATOGRAM OF TISSUE BLANK



EXTRACTED ION CHROMATOGRAM FOR R.T. 13.10 MIN TO 13.25 MIN



II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

See Determinative Method, section H.

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb.

b. No false negatives at 15 ppb or above.

2. Critical Control Points and Specifications

See Determinative Method, section J.2.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Analyze 30 ppb or 1 × standards per day for a two-day period.

ii. Phase II: Fortified samples—Analyze 6 swine liver samples fortified at the regulatory action level of 30 ppb and 2 control samples. Demonstrate that the coefficients of variation for relative ion ratios are within 15%. Repeat experiment for a second day.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

See section F.3.b above.

4. Intralaboratory Check Samples

See Determinative Method, section J.4.

5. Sample Acceptability and Stability

See Determinative Method, section J.5.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	Each set must contain a tissue blank, a spiked tissue fortified at 30 ppb as well as at the highest concentration found in the suspect sample, and all positive samples found above the concentration of interest from the determinative method.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): 30 ppb.

CHLORINATED HYDROCARBON ANALYSIS

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 5
E. Extraction Procedure 7
F. Analytical Quantitation 9
G. [Reserved]
H. Hazard Analysis 10
I. [Reserved]
J. Quality Assurance Plan 12

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Because chlorinated hydrocarbon pesticides are stored in fat or fatty tissue, and tolerances are set for these residues in fat, all such analyses are reported on the basis of the extracted or rendered fat.

The sample is dissolved in petroleum ether and extracted with acetonitrile. The acetonitrile extracts are diluted with water to partition the pesticide residues into petroleum ether. The residues are purified by chromatography on a Florisil column, eluting with a mixture of petroleum and ethyl ethers. Residues in the concentrated eluates are measured quantitatively by gas-liquid chromatography, followed by confirmation by mass spectrometry (GC/MS).

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Filter tubes: Approximately 22 mm × 200 mm, with a short delivery tube and a coarse fritted plate (a glass wool plug can also be used).

b. Kuderna-Danish concentrators: 500 mL, with 5 or 10 mL volumetric receiving flasks or graduated tubes (Kolmer or Mills types: Kontes Glass Company, Vineland, NJ 08360; Catalog No. K-570000, K-621400, or equivalent).

c. Alternate concentrator: If the solutions can be made to a volume of 25 mL, an Erlenmeyer flask with a 24/40 standard taper joint with a three-ball Synder column may be used.

d. Chromatographic columns: 22 mm id × 300 mm, with Teflon stopcocks and coarse fritted plate.

e. Alternate chromatographic columns: 22 mm id × 300 or 400 mm, without stopcocks, with coarse fritted plate. (When this column is used, it is topped with anhydrous sodium sulfate powder for flow control.)

f. Separatory funnels: Teflon stopcocks, 125 mL and 500 mL.

2. Instrumentation

a. Gas liquid chromatograph: Consists of an electron capture detector, power supply, electrometer and 1 mv recorder, on-column injection system, and all-glass column in oven controlled to ± 0.1° C.

b. Column: Supelco 1.5% SP-2250 / 1.95% SP-2401 on 100/120 Supelcoport. Condition column according to manufacturer's specifications.

c. Alternate columns: 6' × 4 mm (approximately) packed with 1.5% OV-17 and 1.95% OV-210 on 100-120 mesh Chromsorb VHP or equivalent, or 1.0% OV-17 and 4.0% OV-210 on 100-120 mesh Gaschrom Q. The column should be conditioned at 250° C. Other equivalent liquid phases and support may be used. (If this column is used, the 6% and 15% elutions may be combined.)

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent and Solution List

a. Acetonitrile, pesticide grade, Burdick & Jackson, saturated with petroleum ether: Saturate CH_3CN with petroleum ether (1.d). A small layer of petroleum ether should be just visible on the CH_3CN.

b. Eluting solvent, 15%: Dilute 150 mL ethyl ether (1.c) to 1 L with petroleum ether (1.d).

c. Ethyl ether: Pesticide grade, Burdick & Jackson.

d. Petroleum ether: Pesticide grade, Burdick & Jackson, 30-60° C, boiling range.

e. Sodium sulfate: Anhydrous granular and anhydrous powder.

f. Aluminum oxide: Al_2O_3G, available from Warner-Chilicott Laboratories, Instruments Division, 200 South Garrad Blvd., Richmond, CA 94801. Al_2O_3-G neutral, available from Merck & Co., Rahway, NJ 07065.

g. Developing solvents.

i. n-Heptane, commercial or technical grade (no purification required).

ii. n-Heptane, commercial or technical grade, containing 2% acetone, reagent grade.

h. Chromogenic agent: Dissolve 0.100 g $AgNO_3$ in 1 mL H_2O, add 20 mL 2-phenoxyethanol (Practical Eastman Organic Chemicals), dilute to 200 mL with reagent grade acetone, add 1 small drop of 30% H_2O_2 and mix. Store in dark overnight and decant into spray bottle. Discard after four days.

i. Florisil: 60/100 pesticide residue (PR) grade, activated at 1250° F (675° C), available from the Floridin Co. When 1250° F activated, Florisil is obtained in bulk. Transfer immediately after opening to 500 mL glass jars or bottles with glass-stoppered or foil-lined screw-top lids, and store in the dark. Heat $\geq$ 5 hours at 130° C before use. Store at 130° C in glass-stoppered bottles or in a desiccator at room temperature and reheat at 130° C after two days.

2. Purity of Solvents

Some solvents, sold as suitable for use as received, are available from many manufacturers, and are usually identified as Nanograde or chromatographic grade. All solvents, however, must be tested prior to use to determine whether purification is required. Electron capture gas chromatography requires the absence of substances causing any detector response from the following test:

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

Place 300 mL of solvent in the Kuderna-Danish concentrator fitted with a three-ball Synder column and a calibrated collection tube and evaporate to 5 mL. Inject 5 μL of concentrate into the gas chromatograph using operating conditions described in F.1. The concentration must not cause recorder deflection greater than 1.0% from baseline for 2 to 60 minutes after injection.

Those solvents requiring purification must be distilled in an all-glass apparatus. However, if Nanograde or chromatographic grade solvents are found to require purification, they should be returned to the supplier with a request for replacement since their high cost is predicated upon purity.

3. Cautionary Note

Ethers (excluding petroleum ether) containing unsafe concentrations of peroxides can detonate when they are distilled or concentrated. No quantity of ether should be distilled or concentrated before the following test is performed:

Shake 1 mL of a freshly prepared saturated solution of KI with 9 mL of ether in a 25 mL glass-stoppered cylinder. Any yellow color indicates a concentration of peroxide greater than 0.005%.

Such a concentration is dangerous, and ether containing this level of peroxide should be discarded or returned to the supplier with a request for an exchange. Prior to discarding or returning the ether, it should be deactivated as follows:

Add 30 mL of a 30% (w/v) aqueous ferrous sulfate solution per liter of ether. Use caution, because this reaction may be vigorous if the ether contains a high concentration of peroxides.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

All FSIS laboratories are to procure chlorinated hydrocarbon standards from:

U. S. Environmental Protection Agency
Director, Environmental Monitoring Support Laboratory
Office of Research & Development
Cincinnati, OH 45268

2. Preparation of Standards

a. Stock standard (0.4 mg/mL): Accurately weigh 10 ± 0.1 mg of each compound of interest into a separate 25 mL flask. Dissolve and dilute to volume with iso-octane.

b. Gas-liquid chromatography: The standards from 2.a are diluted so that a μL injection will contain approximately 0.05 nanogram of heptachlor epoxide and will give approximately 50% of full scale deflection on the recorder. The concentrations of all other insecticides should be adjusted accordingly: Lindane and BHC should be approximately 0.5 of the heptachlor epoxide; dieldrin, endrin, and DDE should be 1.5 times; DDD should be about 2 times; DDT should be about 4 times; and methoxychlor should be about 8 times. This is approximate and must be determined for each gas-liquid chromatograph used.

c. Gas-liquid chromatography mixture standards: After retention time and response have been determined for the separate insecticides, prepare mixtures so that the peaks will not interfere with each other, e.g., HCB, α-BHC, lindane, heptachlor, aldrin, heptachlor epoxide, p-p′ DDE, dieldrin, endrin, p-p′ TDE, p-p DDT, mirex, and methoxychlor. The concentrations should be such that a 5 μL injection produces a 1/2 scale recorder deflection.

d. Florisil: The columns are prepared as in the clean-up technique, and prewashed with 100 mL petroleum ether. A 1 mL aliquot of mixed pesticides containing 0.2 μg lindane, 0.4 μg heptachlor epoxide, 0.6 μg dieldrin, and 0.6 μg endrin is placed on the column. The columns are eluted as in the clean-up technique. The eluate is concentrated, transferred to 25 mL volumetric flasks, and made to volume with petroleum ether. 1 mL of the mixed pesticides is also diluted to 25 mL with petroleum ether in a volumetric flask. Inject 5 μL of the standard and of the eluate into the gas chromatograph. The recovery of all the pesticides when compared to the standard must be at least 95% when the 15% eluate is used.

DETERMINATIVE METHOD

D. STANDARDS (Continued)

3. Storage Conditions	Store standards and various standard dilutions in freezer.
4. Shelf Life Stability	Prepare working standards at least monthly, more frequently if analysts have indications of changing concentrations. Stock standards should be checked if quantitation problems persist and prepared fresh if found to be at improper concentration.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Fatty tissue.

i. Pass chilled sample through food chopper twice. If dry ice is available, add small quantities to fat to maintain a low temperature.

ii. Plug a powder funnel moderately tight with sylanized glass wool and place approximately 100 g of the comminuted fat in the funnel.

iii. Position the funnel in a beaker and render at 100-110° C until the fat ceases to drop.

b. Muscle tissue.

i. Pass sample through food chopper three times. Grind suitable quantity with anhydrous Na_2SO_4 to combine with moisture in sample.

ii. Transfer granular mixture to centrifuge bottle, add 100 mL petroleum ether, shake vigorously, and centrifuge about 5 minutes at about 1500 rpm.

iii. Pour off solvent layer into beaker and re-extract residue twice with 50 mL portions of petroleum ether. Evaporate combined extracts to obtain fat.

NOTE: All chlorinated hydrocarbon residues are reported on the basis of the rendered or extracted fat.

2. Acetonitrile Partitioning

a. Weigh 3 g of melted fat into a 50 mL beaker, add 10 mL petroleum ether, and transfer to a 125 mL separatory funnel. Fortify each sample with an amount of aldrin as an internal standard that results in an approximate 50% scale deflection.

NOTE: An enhanced peak for aldrin may indicate the presence of ronnel.

b. Use an additional 15 mL in small portions to rinse the beaker for a quantitative transfer of the fat.

c. Add 25 mL of acetonitrile saturated with petroleum ether and shake for 1 min.

d. Let layers separate. (Adequate time must be allowed to obtain a good separation.)

e. Drain the bottom (acetonitrile) layer into a 500 mL separatory funnel containing 300 mL of 4% Na_2SO_4 and 100 mL petroleum ether.

f. Extract the petroleum ether solution in the 125 mL separatory funnel with 3 additional portions of acetonitrile saturated with petroleum ether, shaking vigorously 1 min each time. Combine all extracts in the 500 mL separatory funnel.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

g. Stopper the 500 mL separatory funnel, invert, vent pressure, and shake gently 1 minute to transfer the pesticides to petroleum ether.

h. Let layers separate cleanly and discard lower aqueous layer.

i. Wash the petroleum ether in 500 mL separatory funnel with two 50 mL portions of 4% Na_2SO_4. Stopper, invert, and vent after each addition.

CAUTION: Occasionally excessive pressure will build up.

j. Discard washings and draw off petroleum ether layer through a 2-in column of anhydrous granular Na_2SO_4 in the filter tube into a 250 mL Erlenmeyer flask.

k. Rinse the separatory funnel and the column with two 10 mL portions of petroleum ether.

l. Evaporate combined extract and rinses to ca 10 mL in a Kuderna-Danish or alternate concentrator.

3. Florisil Column Chromatography

a. Prepare 22 mm id Florisil column containing 100 mm after packing of activated Florisil topped with about 1/2-inch of anhydrous sodium sulfate powder (110 mm should pack to 100 mm).

b. Prewet column with 40-50 mL petroleum ether.

c. Transfer petroleum ether extract to column, letting it pass through the column at a rate not to exceed 3 mL/min (optimum rate is approximately 2.5 mL/min).

d. When the last of the petroleum ether has sunk into the column, change receivers and elute with 200 mL of 15% ethyl in petroleum ether.

e. Concentrate each eluate to 5 mL using the Kuderna-Danish or alternate concentrator. Transfer each eluate quantitatively to a 25 mL volumetric flask and make to volume with petroleum ether. When the elution from the Florisil column is held to the low rate specified, the insecticides are eluted quantitatively from the column and both eluates are suitable for gas-liquid chromatography.

f. The 15% eluate contains aldrin; BHC; DDE; DDD (TDE); o,p-DDT and p,p'-DDT; heptachlor; heptachlor epoxide; lindane; methoxychlor; HCB; Mirex; oxychlordane; dieldrin; and endrin.

NOTE: Florisil activity varies from lot to lot. Therefore, the elution volume must be determined empirically for each lot; it will not always be 200 mL.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Gas Chromatography Analysis

a. Operating conditions for 10% OV-101 or the mixed phase column.

Injection temperature	225° C
Column temperature	185-200° C
Detector temperature	325° C (maximum if Ni^{63} detector is used)
Carrier gas flow	120 mL/min
Carrier gas	argon/methane (5%)

Detector voltage, instrument settings must be determined for each chromatograph to obtain the maximum sensitivity.

b. Check the linearity of the system. Determine the individual retention times of the insecticides. *Make calculations only from peaks that are in the linear range.*

c. Inject a suitable aliquot (3-8 μL) of the eluates from the 25 mL volumetric flask into the chromatograph using a 10 μL syringe. If peaks are out of the linear range, re-inject using a smaller aliquot or dilute to bring the response into the linear range.

d. Tentatively identify residue peaks on the basis of retention time. Measure areas under the peak or peaks from the unknown and compare to the areas obtained from known quantity of the standard insecticide. For most accurate measurement, unknown and standards should be of similar size (i.e., $\pm$ 15%) and in the linear range of the detector used.

e. Chromatograph the standard pesticide immediately after the sample. Alternatively, it is possible to use only peak heights with a slight loss in accuracy, but good judgement is required, and it is *very* important that all measurements be in the linear range of the detector.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Chlorinated Hydrocarbon Analysis
2. Required Protective Equipment	Safety glasses, plastic gloves, heat-resistant gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
E.1.b. Petroleum ether	Large volumes of petroleum ether are flammable and may induce nausea.	This step should be performed in an efficient fume hood away from electric heaters. Evaporation is accomplished by use of a steam bath.
E.2 Petroleum ether Acetonitrile	Pet ether (see above). Acetonitrile is also flammable. Vapors are irritating and may be absorbed through the skin, producing toxic effects.	Work in an efficient fume hood and use the required plastic gloves when adding solvent and shaking the funnels.
E.3 Petroleum ether Ethyl ether	Pet ether (see above). Ethyl ether is extremely flammable and explosive. Vapors can be very irritating to respiratory system.	All work must be done in an efficient fume hood. The ethers are evaporated on a steam bath using Kuderna-Danish condensers or an equivalent. Each lot of ethyl ether should be checked for peroxides.
F.1 EC detector	Radioactive.	Needs to be checked for leakage of radioactivity every 6 months. Its exhaust should be vented to an efficient fume hood.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS (Continued)

4. Disposal Procedures	Acetonitrile/ aqueous waste	May produce toxic effects.	Partially evaporate in the hood and store the remainder in a nonchlorinated solvent collection can until disposed of by a contractor or in-house specialist.
	Others	See H.3	Completely evaporate in the fume hood.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
Aldrin	80-110	12	20
BHC	80-110	12	20
Chlordane	80-110	12	20
Dieldrin	80-110	12	20
DDT	80-110	12	20
DDE	80-110	12	20
TDE (DDD)	80-110	12	20
Endrin	80-110	12	20
Heptachlor	80-110	12	20
Heptachlor epoxide	80-110	12	20
Lindane	80-110	12	20
Methoxychlor	80-110	12	20
Toxaphene	80-110	12	20
PCB's	75-110	12	20
HCB	80-110	12	20
Mirex	80-110	12	20
Nonachlor	80-110	12	20
Strobane	80-110	12	20

2. Critical Control Points and Specifications

Plastic sample bags—check for interfering peaks.

Record	*Acceptable Control*
Chromatograms (See note on chromatograms below.)	Any GLC and column that separates for quantitation all compounds of interest (resolution). Each chromatogram to be timed for one minute past the time coinciding with the retention time for the last eluting compound of interest. (For PCB's, an OV101 or DC200 column must be used.)
Solvent, lot no., source, date checked, deactivation (if required), and signature.	No peroxides present in ethyl ether.
Solvent, lot no., source, date checked, findings, and signature.	No deflection > 1.0% full scale from baseline from 2-60 min after injection.
Each individual chlorinated hydrocarbon, its concentration and scale deflection, date of test, and signature.	5 μL injection containing 0.05 ng heptachlor epoxide giving about 50% full scale deflection, amounts of other chlorinated hydrocarbons giving about the same deflection.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

Record	*Acceptable Recovery*
Time and temperatue for Florisil activation.	Activated at 1250° F (650° C) > 2 hours if not already done by supplier, followed by ≥ 5 hours at 130° C and storage in desiccators. ≥ 5 hours at 130° C must be repeated for each batch at least every 2 days.
Amount (if any) of deactivation with water to achieve desired recoveries.	All compounds with 95% recovery through the column. (80-110% through the entire procedure.)
Rendering oven temperature.	100-110° C.
Sample weight.	3.0 ± 0.1 g fat (Guidebook) ≤ 3.0 ± 0.1 g fat (AOAC)
Internal standard.	Fortify each sample with an amount of aldrin that produces an approximate 50% scale deflection. (Recovery must be 80-110% or sample must be reanalyzed.)
Column elution.	2.5 ± 0.15 mL/min (Guidebook); © 5 mL/min for PCB's (AOAC). 200 mL 6% for PCB's or if GC system used resolves all compounds of interest, only 200 mL 15% elution solvent.

NOTE: Each set of samples or day's run must be represented by properly labeled chromatograms containing at a minimum the following items:

- Chromatogram stamp with the following information:

  Operator . . . . . Date . . . . . .

  COLUMN: No . . . . . Length . . . . . Dia . . . . . .

  Coating . . . . . Concn . . . . . .

  Support . . . . . Mesh . . . . . .

  Theoretical Plates . . . . . .

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

TEMP: Col: Init . . . . . °C Final . . . . . °C

Rate . . . . . °C/min Det . . . . . °C Inj . . . . . °C

CARRIER GAS: . . . . . Rate . . . . . mL/min

Pressures: Inlet . . . . . Outlet . . . . . .

Gas(Type) . . . . . mL/min Gas(Type) . . . . . mL/min

DETECTOR: . . . . . ; . . . . . ma . . . . . volts

Temperature . . . . . .

Scavenger . . . . . Rate . . . . . mL/min

Sens . . . . . Rec. range . . . . . mv

Response . . . . . Area/Unit Wt. of Compound

SAMPLE: . . . . . Size . . . . . .

Solvent . . . . . Final Concn . . . . . μg/mL Vol . . . . . mL

CHART: Speed . . . . . .

- Each standard labeled with the following items:

  Standard Identification
  Concentrations of compounds of interest
  Peak identifications
  Microliters injected
  Applicable measurements

- Each sample labeled with the following items:

  Identification
  Weight
  Dilutions
  Microliters injected
  Identification of chlorinated hydrocarbon peaks
  Applicable measurements
  Quantitative calculations

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—At least 3 levels, 3 replicates each, for each compound of interest (hand-injected or by auto-injector if official analyses will be performed in that manner).

(a) At minimum proficiency level.

(b) At twice minimum proficiency level.

(c) At one-half minimum proficiency level.

ii. Phase II: Fortified samples must include at least 3 levels, with 3 replicates each, for each compound of interest. Each set includes reagent blanks and tissue blanks.

(a) At minimum proficiency level.

(b) At twice minimum proficiency level.

(c) At one-half minimum proficiency level.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples plus controls from FSIS Science & Technology Western Laboratory.

(b) Report analytical findings to Chemistry Division.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Minimum of 1 per week per analyst, more for new or trainee analysts.

ii. Blind samples—require ''dummy forms.''

iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Running average difference between replicates.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

(c) All percent recoveries.

(d) Internal standard recoveries.

(e) For all recoveries: the running average, standard deviation, and coefficient of variation of the last 10 acceptable recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Fat, extracted fat adipose tissue.

b. Sample receipt size, minimum: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests and reserve sample.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage.

i. Time: Indefinite (chlorinated hydrocarbons); estimated 1 month (organophosphates).

ii. Condition: Frozen (all compounds).

6. Sample Set

Each set processed on the same day should include:

a. Tissue blank.

b. Blank tissue fortified at tolerance level for compound of interest.

c. Sample(s).

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

7. Sensitivity

a. Lowest detectable limit (LDL), ppm:

0.01: BHC, dieldrin, heptachlor, heptachlor epoxide, lindane, HCB.
0.02: aldrin, DDE.
0.03: endrin.
0.04: DDT, TDE (DDD), mirex.
0.05: nonachlor.
0.15: chlordane, methoxychlor.
0.30: PCB's.
0.50: toxaphene, strobane.

b. Minimum proficiency level (MPL), ppm:

0.10: aldrin, BHC, dieldrin, DDE, endrin, heptachlor, heptachlor epoxide, lindane, HCB, mirex.
0.15: DDT, TDE (DDD), nonachlor.
0.30: chlordane.
0.50: methoxychlor, PCB's.
1.00: toxaphene, strobane.

CHLORINATED HYDROCARBONS AND PCB RESIDUES IN FATTY FOODS

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 7
G. [Reserved]
H. Hazard Analysis 8
I. [Reserved]
J. Quality Assurance Plan 9

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Chlorinated hydrocarbons are extracted and separated by elution from fat in small glass columns filled with partially deactivated alumina. The eluate is carefully evaporated to a workable volume and an aliquot injected into a gas chromatograph for detection and quantitation.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. 50 mL (or other suitable size) ground glass (19/22) Erlenmeyer flask, Kontes #K-296510, or equivalent.

b. 19/22 ground glass toppers, Kontes #K-850500, or equivalent.

c. Micro column: 200 mL plain chromaflex columns with Teflon stopcock (Kontes #K-420280-213) or 12 × 300 mm fritted glass columns with Teflon stopcock, or equivalent.

d. Glass wool (silanized).

e. 10 mL leur-lok syringe with 2″ or 4″ 17-gauge needle.

f. Explosion-proof blender, Waring Model E-1, or equivalent.

g. Concentrator tubes, Kdurna Danish, or equivalent.

h. 25 mL volumetric flask.

2. Instrumentation

Gas chromatograph: Equipped with electron capture detector and a column of choice, having at least 1,500 theoretical plates for aldrin and the ability to separate all compounds of interest.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Alumina-Fisher Alumina (catalog #A950), Wolm Alumina, or ICN Alumina (catalog #408420), neutral, Brockman Activity I, 80/200 mesh, or equivalent. Treat alumina in an 800° C furnace for a minimum of 4 hr in an open container. Store in a 130° C oven in an open container. Deactivate by cooling to room temperature in capped container, adding a percentage of distilled water, the percentage of which has been predetermined to give an 80-110% recovery through the column of the chlorinated hydrocarbon of interest, by weight. Recap and shake vigorously and allow to equilibrate overnight or at least for 4 hr before use. Deactivate only the amount to be used each day.

b. p-Methoxyazobenzene (4 phenlyazo-anisole): Columbia Organic Chemicals Co., Inc., Columbia, SC 29220, or equivalent. Dissolve 0.3 g in 100 mL hexane.

c. Hexane: distilled in glass, Burdick and Jackson, or equivalent.

d. Acetone: distilled in glass, Burdick and Jackson, or equivalent.

e. Petroleum ether: distilled in glass, Burdick and Jackson, or equivalent.

f. ''Blank'' fat.

g. Standard chlorinated hydrocarbon solutions (concentration as appropriate for instrumentation and samples being tested).

DETERMINATIVE METHOD

D. STANDARDS

1. Source	All FSIS laboratories are to procure PCB standards from one source: Food and Drug Administration Division of Chemical Technology, HFF-420, 200 C Street, S.W. Washington, DC 20204
2. Storage Conditions	Store standards and various standard dilutions in freezer.
3. Shelf Life Stability	Prepare working standards at least monthly, more frequently if analysts have an indication of changing concentrations.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. For high-fat samples, grind or chop and heat-render at 100°-110° C.

b. For low-fat samples, grind and solvent-extract by blending approximately 200 g sample with 1 + 1 acetone/petroleum ether in an explosion-proof blender. Filter tissue and solvent through glass wool plug in a funnel into a 400 mL beaker and allow layers to separate. Decant petroleum ether layer containing the fat into 100 mL beaker. Add a few glass beads and evaporate solvent on a steambath.

2. Column Preparation

a. Fill the glass columns (refer to section B.1, item c), fitted with small glass wool plug, 3/4 full with petroleum ether or hexane, depending upon column type.

b. Add 10 g of prepared alumina and allow to settle.

c. Drain just to top of alumina in column.

d. Collect petroleum ether or hexane in a suitable size Erlenmeyer flask. Rinse flask with this solvent and discard. Replace flask under column.

3. Sample Extraction

a. Determine amount of elution solvent necessary to separate chlorinated hydrocarbons from the lipids in the sample by dissolving 1 mL of dye solution (refer to section C, Reagent and Solution List, item b) in 0.1-0.2 g ''blank'' fat.

b. With a syringe, transfer this ''dyed'' sample to a prepared column with small portions (totaling 5 mL) of petroleum ether or hexane.

c. Allow to drain into column with a flow rate of 1 drop/sec, collecting eluate in receiving flask.

d. Fill the column with more petroleum ether or hexane as initial 5 mL penetrates into alumina and adjust flow rate to 1 drop/sec.

e. The visible dye will separate from the fat as it flows down the column in a bright orange band, which may disperse slightly near the bottom. When this band has eluted, masure the volume required to elute the dye band. this is the amount usually sufficient to elute the chlorinated hydrocarbons from the column separated from the fat. (Unusual amount is approximately 35 mL.) Slightly more elution solvent may be required for some chlorinated hydrocarbons.

NOTE: Steps a-e may be eliminated, but there is a danger of ruining the column in the gas chromatograph if this is done. It is preferable to conduct these steps one time with each set of samples.

f. Quantitatively weigh 0.1-0.2 fat into aluminum dish or small beaker, or weigh 2.5 g rendered fat into 25 mL volumetric flask and dilute to volume with hexane or petroleum ether. For quality control purposes, add a known amount of aldrin to each sample as an internal standard.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

g. Rinse fat sample (or 1 mL of fat solution prepared in step f) into a prepared alumina column with syringe, using about 5 mL petroleum ether or hexane, and allow to drain to near the alumina surface, with care not to allow the column to go dry.

h. Rinse sample beaker or dish with additional petroleum ether or hexane and add rinses to column. Collect eluate in a suitable size flask for volume determined in step e. The rinses comprise part of the predetermined solvent volume. If a concentrator tube with an attached reservoir (Kdurna Danish) is used to collect the eluate, the transfer in step j below may be avoided.

i. After elution of column, use syringe to rinse tip of column and neck of flask with a small amount of petroleum ether or hexane.

j. Evaporate solvent to 2-3 mL using steam bath. Transfer to a concentrator tube and concentrate nearly to dryness using a stream of filtered nitrogen or air. Do not use heat, and do not allow sample to go to dryness. If residue shows enough lipid material to cause chromatographic problems, start over—do not go to section F.

4. Reference Clacys, Robert R., and Inman, Roderick D., *JAOAC*, 57 (1974).

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

General Operation	Dilute residue wiwth 1-4 mL hexane or petroleum ether and inject into appropriate gas chromatograph for detection and quantitation.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Micro Alumina Column Screening for Chlorinated Hydrocarbons and PCB Residues in Fatty Foods

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat, heat-resistant gloves.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
E.1.b. Acetone Petroleum ether	Both acetone and petroleum are very flammable and the vapors may induce nausea.	Perform all work in an efficient fume hood away from electric heaters. Use steam bath for evaporations.
E.2, E.3 Hexane Petroleum ether	These chemicals are very flammable and can produce toxic effects.	(Same as above.) Use of evaporator tubes with Kuderna-Danish type condensers will further minimize exposure to these hazards.

4. Disposal Procedures

Hexane Petroleum ether	See above.	Evaporate these solvents in a fume hood.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compounds	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
PCB 1242 PCB 1248 PCB 1254 PCB 1260	75-100	12	20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Chromatograms (See note on chromatograms below.)	For PCB's, an OV101 or DC200 must be used.
Solvent, lot no., source, date checked, deactivation (if required), and signature.	No peroxides present in ethyl ether.
Solvent, lot no., source, date checked, findings, and signature.	No deflection > 1.0% from base line from 2-60 min after injection of concentrated solvent.
Amount (if any) of deactivation with water to achieve desired recoveries.	95% recovery through column. (75-100% through the entire method.)
Rendering oven temperature.	< 110° C.
Sample weight.	≤ 3.0 g ± 0.1 g fat.
Elution solvent.	200 mL 6% at < 5 mL/min flow rate.

NOTE: Each set of samples or day's run must be represented by properly labeled chromatograms containing at a minimum the following items:

- Chromatogram stamp with the following information:

  Operator . . . . . Date . . . . . .

  COLUMN: No . . . . . Length . . . . . Dia . . . . . .

  Coating . . . . . Concn . . . . . .

  Support . . . . . Mesh . . . . . .

  Theoretical Plates . . . . . .

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

TEMP: Col: Init . . . . . °C Final . . . . . °C

Rate . . . . . °C/min Det . . . . . °C Inj . . . . . °C

CARRIER GAS: . . . . . Rate . . . . . mL/min

Pressures: Inlet . . . . . Outlet . . . . . .

Gas(Type) . . . . . mL/min Gas(Type) . . . . . mL/min

DETECTOR: . . . . . ; . . . . . ma . . . . . volts

Temperature . . . . . .

Scavenger . . . . . Rate . . . . . mL/min

Sens . . . . . Rec. range . . . . . mv

Response . . . . . Area/Unit Wt. of Compound

SAMPLE: . . . . . Size . . . . . .

Solvent . . . . . Final Concn . . . . . μg/mL Vol . . . . . mL

CHART: Speed . . . . . .

- Each standard labeled with the following items:

  Standard Identification
  Concentrations of compounds of interest
  Peak identifications
  Microliters injected
  Applicable measurements

- Each sample labeled with the following items:

  Identification
  Weight
  Dilutions
  Microliters injected
  Identification of chlorinated hydrocarbon peaks
  Applicable measurements
  Quantitative calculations

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards—At least 3 levels, 3 replicates each, for each PCB of interest (hand-injected or by auto-injector if official analyses will be performed in that manner).

(a) At minimum proficiency level.

(b) At twice minimum proficiency level.

(c) At one-half minimum proficiency level.

ii. Phase II: Fortified samples must include at least 3 levels, with 3 replicates each, for each PCB of interest. Each set includes reagent blanks and tissue blanks.

(a) At minimum proficiency level.

(b) At twice minimum proficiency level.

(c) At one-half minimum proficiency level.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples plus controls from FSIS Science & Technology Western Laboratory.

(b) Report analytical findings to Chemistry Division.

b. Acceptability criteria.

Refer to Section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Minimum of 1 per week per analyst, more for new or trainee analysts.

ii. Blind samples—require ''dummy forms.''

iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Running average difference between replicates.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

(c) All percent recoveries.

(d) For all recoveries: the running average, standard deviation, and coefficient of variation of the last 10 acceptable recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Fat, extracted fat adipose tissue.

b. Sample receipt size, minimum: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests and reserve sample.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage.

i. Time: Indefinite.

ii. Condition: Frozen.

6. Sample Set

Each set processed on the same day should include:

a. Tissue blank.

b. Blank tissue fortified at tolerance level for compound of interest.

c. Sample(s).

7. Sensitivity

a. Lowest detectable limit (LDL), ppm: 0.30.

b. Minimum proficiency level (MPL), ppm: 0.50.

ORGANOHALIDES IN ANIMAL FAT (GPC)

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 9
G. [Reserved]
H. Hazard Analysis 13
I. [Reserved]
J. Quality Assurance Plan 14

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Liquified animal fat is dissolved in methylene chloride:cyclohexane (1:1) solvent. Residues are purified by gel permeation chromatography (GPC) and identified and measured by GLC-EC detection. This method has been collaboratively studied, with acceptance by both AOAC and FSIS for compounds listed in 2.a and by FSIS for compounds listed in 2.b.

2. Applicability

a. Compounds for which method has been collaborated.

aldrin	dieldrin	heptachlor
alpha BHC	o,p′-DDT	heptachor epoxide
lindane	p,p′-DDT	hexachlorobenzene
cis-chlordane	p,p′-DDE	methoxychlor
trans-chlordane	p,p′-TDE	mirex
oxychlordane	endrin	

b. Compounds to which method applies for quantitation.

toxaphene	halowaxes	nonachlor
coumaphos-O	coumaphos-S	ronnel
phosalone	kepone	captan
linuron	endosulfan I	endosulfan II
chlorpyrifos	polychlorinated biphenyls	stirophos (Gardona®)
carbophenothion (Trithion®)	chlorfenvinfos (Supona®)	

c. Compounds for identification only.

dichlofenthion	chlordene	o,p′-TDE
beta BHC	leptophos	o,p′-DDE
delta BHC		

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Solvent concentration apparatus: Rotary evaporation system or steam bath. Optional: Autovap Model 606, Analytical Biochemistry Laboratories, Inc., P.O. Box 1097, Columbia, MO 65205.

b. Convection oven: Able to maintain a temperature of 80° C ± 3° C.

c. Bio Beads SX-3 resin, 200-400 mesh. Analytical Biochemistry Laboratories, Inc., P.O. Box 1097, Columbia, MO 65205.

d. Pear-shaped flasks: 100 mL. Kontes.

e. Volumetric Class A flasks: 10 mL.

f. Reverse flow chromatography column: 2.5 × 60 cm. Kontes.

g. Filters: Millipore LC-10.

h. Syringe: 10 mL, glass.

2. Instrumentation

a. Gel permeation chromatographic system (GPC): AutoPrep 1002A Gel Permeation Chromatograph. Analytical Biochemistry Laboratories, Inc., P.O. Box 1097, Columbia, MO 65205, or equivalent.

b. Gas chromatograph-EC detector (Ni^{63}): See AOAC Official Methods, Sec. 29.008.

c. GC column: DB-608 fused silica, 30 mm × 0.53 mm ID megabore column, J&W Scientific, Inc., Folsom, CA, Catalog #125-133, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Solvents: Hexane, methylene chloride, 224-bimethyl pentane—glass-distilled, capillary GC and GC-MS quality.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

All FSIS laboratories are to procure chlorinated hydrocarbon standards from one source:

U. S. Environmental Protection Agency
Director, Environmental Monitoring Support Laboratory
Office of Research and Development
Cincinnati, OH 45268

PCB standards may be obtained from:

Food and Drug Administration
Division of Chemical Technology, HFF-420,
200 C Street, S.W.
Washington, DC 20204

2. Preparation of Standards

a. Stock standard (0.4 mg/mL): Accurately weigh 10 ± 0.1 mg of each compound of interest into a separate 25 mL flask. Dissolve and dilute to volume with iso-octane.

b. Fortification standard: 1.5 μg/mL for linuron, coumaphos-O, and coumaphos-S; 0.5 μg/mL for all other pesticides.

   i. 1.5 μg/mL solution: Pipet 0.375 mL linuron, coumaphos-O, and coumaphos-S stock standard solutions into a 100 mL volumetric flask and dilute to volume with iso-octane.

   ii. 0.5 μg/mL solution: Pipet 0.125 mL of each compound from stock standard solutions into a 100 mL volumetric flask and dilute to volume with iso-octane.

c. Working standard (for GC): Pipet 0.400 mL of the fortification standard (step 2.b) into a 10 mL volumetric flask and dilute to volume with iso-octane. This will yield 0.06 μg/mL for linuron, coumaphos-O, and coumaphos-S and 0.02 μg/mL for all other pesticides.

3. Storage Conditions

Store standards and various working solutions in the freezer.

4. Shelf Life Stability

Prepare working solutions at least monthly or more frequently if analysts have indication of changing concentrations. Stock standards should be checked if quantitation problems persist and prepared fresh if found to be an improper concentration.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Place approximately 40 g of representative animal fat sample in a glass funnel (8.0 cm) with a silanized glass wool plug.

b. Place funnel in flint glass bottle or 250 mL beaker at ≤ 80° C and collect 5-10 g of fat.

c. Mix thoroughly.

2. Column Preparation

a. Place 60 g of BioBeads SX-3 into a 500 mL beaker.

b. Cover the resin with at least 2 inches of methylene chloride:cyclohexane (50:50) mixture and allow it to stand overnight to swell the gel.

c. Decant off fines and add fresh solvent. Allow to stand 30 minutes and again decant off fines. Repeat two more times.

d. Secure the upper plunger assembly (short plunger) in the tube by inserting the plunger and turning it clockwise until snug.

CAUTION: This end of the column is the exit and the plunger should not be loosened again. Adjustment after the resin is packed may result in wrinkling the Teflon cloth seal and allow the BioBeads to escape into the instrument.

e. Clamp the chromatography tube upside-down on a ring stand and place the line from the upper plunger assembly into a waste beaker below the column.

f. Transfer the swelled resin to the column as a slurry with the solvent mixture.

g. Allow the resin to settle and the excess solvent to drain from the column until the levels of solvent and BioBeads are equal.

h. Insert the other plunger assembly (the long plunger) into the tube until it touches the gel; tighten the plunger by turning it clockwise until snug.

i. Connect the column input and output lines together and install the column onto the pumping system.

j. Connect the column input line to the correct line from the sample introduction valve.

k. Place a glass beaker under the column output line.

l. Turn the pump on and allow it to run until solvent runs out the column output line.

m. Hold the glass beaker with the column output line in it next to a light and check to ensure that no BioBeads are escaping from the column.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

n. If the column has voids or air pockets after pumping for 230 minutes, loosen the inlet plunger (lower long plunger) and compress the column.

o. Retighten the inlet plunger and continue pumping. Normal pressure is about 5-15 psi at a flow of 5 mL/min.

2. Column Calibration (Auto-Prep Only)

The elution volumes in section E.4 are used as a guide to determine approximately what volume to collect during fractionation. It is recommended that the volume of eluant from 150 mL to 320 mL be fractionated to ensure residue collection. An initial dump time of 30 minutes, followed by 17 two-minute collect times, will provide a margin before and after the usual elution volume. To autofractionate, proceed as follows:

a. Turn the ''power on/off'' switch on the control module to the ''on'' position.

b. Press the ''pump enable'' switch to start the pump set at a flow of 5 mL/min.

c. Set the ''terminal loop'' thumbwheel switch to ''17'' and place collection vessels under the appropriate numbered sample collection tubules.

d. Set the ''wash'' time switch to ''00.''

e. Set the ''collect'' time switch to ''02'' with the ''time base'' switch set at 1 minute.

f. Set the ''dump'' time switch to ''30.''

g. Turn the ''pump off/on'' switch to ''on.''

h. Depress the ''reset'' switch until the ''current loop'' display reads ''00.''

i. Press the ''index'' switch to ''01'' on the ''current loop'' display.

j. Rotate the ''load/run'' valve on the cabinet counter clockwise to the ''load'' position.

k. Inject a minimum of 8 mL of the sample (suspended in GPC solvent) into #1 through the sample introduction port with a 10 mL syringe.

l. ''Index'' to loop ''02'' with the syringe still in position.

m. Remove the syringe and rinse with clean solvent.

n. Rinse loop ''02'' and connecting lines by injecting 8 mL of clean solvent.

o. Push the ''reset'' switch until ''00'' is displayed on the ''current loop'' display. Be sure all loops are full of solvent.

p. Rinse loop ''00'' with 8 mL of clean solvent, rotate the ''load/run'' valve to ''run,'' remove the syringe, and depress the ''auto start switch.'' The ''dump'' time of 30 minutes is loaded into memory.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

q. Now set the ''dump'' time switch to ''00.'' The AutoPrep is now programmed to dump 150 mL and collect 170 mL in 10 mL fractions.

r. The solvent in each 10 mL fraction can be evaporated and reconstituted to 1 mL volume in iso-octane for a qualitative GLC-EC determination. After determining which fractions contain organochlorine pesticides, the collection volume must be decided. Only a small percentage of gamma BHC (lindane) should elute from the column in the 160 mL to 170 mL fraction (shown in E.4).

In an attempt to limit the amount of lipid in the collected fraction, collection should begin as late as possible. This will allow more of the ''late'' eluting lipid to be dumped before collection begins. Finally, a 5 mL margin is added after the last residue-containing fraction to further ensure quantitative recovery.

NOTE: Spare calibrated columns with the ends tightly capped should be stored with solvent. Follow manufacturers' instructions if Auto-VAP is used.

3. Sample Chromatography

a. Weigh 2.0 g ± 0.1 g fat into a 10 mL volumetric flask. Fortifications are made at this point in the procedure (standards in CH_2Cl_2:C_6H_{12}).

Fortify each sample with 0.20 ppm of aldrin as a surrogate standard, which results in an approximate 50% scale deflection. Dilute to 10 mL with CH_2Cl_2:C_6H_{12} (1 + 1) and mix thoroughly. For screening purposes, the fat sample is weighed directly into 16 × 125 mm disposable glass culture tubes. Solvent is added to a predetermined 10 mL mark and the tube is capped with a Teflon-lined cap. Mix by vortexing.

b. Sample fortification: Add 0.8 mL of the fortification standard (D.2.b) to 2 g of the sample. This will yield a recovery rate of 0.6 ppm for linuron, coumaphos-O, and coumaphos-S and 0.2 ppm for all other pesticides.

c. Centrifuge or filter with Millipore LC 10 filters. Use an 8 mL sample to load sample loops on precalibrated GPC. A 5 mL aliquot is placed into the sample loop. Process through GPC using dump/collect times from fractionation procedure and collect eluant in a 100 mL pear-shaped flask. Rotary evaporate to dryness at ≤ 30° C. Transfer residue quantitatively with 10 mL iso-octane or equivalent GLC-EC compatible solvent. Adjust volume under a gentle dry N_2 stream to the volume required; quantitate by GC. (An alternative concentration procedure may be used with an Autovap 600 as specified in the ''Instruction Manual for Autovap 600,'' [September, 1986] pages 4-11 through 4-17.)

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Guideline

Organohalide Elution Profiles Through 60 g SX-3 Styrene Divinylbenzene Gel (ca. 48 cm bed length × 2.5 cm column diameter).

Organochlorine Pesticide	Elution Volume (mL) with CH_2Cl_2:C_6H_{12} (1:1, v:v)
aldrin	160-240
alpha BHC	180-240
gamma BHC (lindane)	170-240
cis-chlordane	160-250
trans-chlordane	160-230
dieldrin	160-230
o,p′-DDT	160-230
p,p′-DDE	160-220
p,p′-TDE	160-220
p,p′-DDT	160-230
endrin	160-220
heptachlor epoxide	160-230
HCB	190-240
methoxychlor	160-230
mirex	160-230
oxychlordane	150-200
toxaphene	160-230
all others	160-240

DETERMINATIVE METHOD

F. ANALTYICAL QUANTITATION

1. Instrumental Settings and Conditions

Gas chromatograph should be equipped with constant current/pulse-frequency electron capture (EC) detector and data system.

Column	See B.2
Injector temperature	230° C.
Detector temperature	325° C.
Carrier gas	Ultrapure He
Flow rate	20 mL/min (calculate linear velocity)
Makeup gas	Argon/Methane
Flow rate	30 mL/min

Splitless mode with valve on for 1.5 min.

Oven temperature program:	
Initial temperature	170° C.
Hold	10 minutes.
Program	3° C/min
Final temperature	260° C.

2. General Operation

a. Retention times, relative to aldrin:

HCB: 0.33	trans-chlordane: 1.84	p,p′-DDT: 2.72
alpha BHC: 0.49	cis-chlordane: 1.88	carbophenothion: 2.76
lindane: 0.66	chlorfenvinphos: 1.90	mirex: 3.22
heptachlor: 0.78	dieldrin: 2.00	methoxychlor: 3.39
aldrin: 1.00	p,p′-DDE: 2.04	phosalone: 3.56
ronnel: 1.06	captan: 2.11	coumaphos-O: 3.90
linuron: 1.26	stirofos: 2.11	coumpahos-S: 4.00
oxychlordane: 1.28	kepone: 2.20	toxaphene: NA
chlorpyrifos: 1.28	endrin: 2.22	PCB 1242: NA
nonachlor: 1.29	p,p′-TDE: 2.44	PCB 1248: NA
heptachlor epoxide: 1.54	o,p′-DDT: 2.44	PCB 1254: NA
endosulfan I: 1.78	endosulfan II: 2.56	PCB 1260: NA

b. All residues of interest must be 50% resolved for quantitation. The following pairs of compounds coelute and if detected must be differentiated by mass spectrometry: oxychlordane/chlorpyrifos; p,p′-TDE/o,p′-DDT; and captan/stirofos.

Chromatographic parameters should be set such that a 20 pg injection of aldrin yields at least a 50% full-scale deflection. Compounds of interest must not be degraded by any part of the GC system.

c. The run time should be 5 minutes greater than the retention time for coumaphos-S. A 1-3 μL injection containing 20 pg of heptachlor epoxide in iso-octane should give approximately 50% full-scale deflection. The concentrations of the other organochloride pesticides in the standard solution can be adjusted to produce recorder deflections similar to heptachlor epoxide. The peak recognition window for the compounds should be $\pm$ 1% of the retention times. Peak widths should be set at 0.01-0.04 min (see section E.4).

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

d. Toxaphene is quantitated using capillary chromatography by measuring total heights (areas) of all peaks common to the sample and to the toxaphene reference standard.

e. Results should be corrected for percent recovery of fortified sample (fat) carried through the entire procedure. Percent recovery used in calculations is the average of the last 10 acceptable recoveries for the pesticide in question.

f. When integrators are used, area counts or peak heights generated by the computer should be monitored regularly for consistency between printout and manual measurements.

g. When not in use, the DB-608 column should be maintained at 230° C.

h. Capillary chromatography with EC detection is used for separation and quantitation of all compounds except PCB's. If PCB's are detected by this procedure, the sample must be reanalyzed and injected onto a GC column packed with 10% OV-101. Quantitation is accomplished by using weight-percent factors for individual GC peaks in the PCB reference material. (See Table 29.02 in the AOAC Book of Methods, 14th Edition.)

3. Gas Chromatography: Identification and Quantitative Measurement

NOTE: Applicable to organochlorine pesticides, organophosphorous pesticides, and polychlorinated biphenyls (PCB's). Method is applicable to PCB residues when present alone in sample. If pesticidal or other compounds are detected in the chromatogram of the PCB's residue, other chemical or physical operations must be applied to eliminate or minimize their interference before PCB's quantitation.

a. Inject suitable aliquot (3-8 μL) of concentrated eluate from Florisil or MgO-Celite column containing amount of compound within linear range into gas chromatograph using 10 μL syringe. Tentatively identify residue peaks on basis of retention times. Measure area or height of residue peaks and determine residue amount by comparison to peak area or height obtained from known amount of appropriate reference material. To ensure valid measurement of residue amount, size of peaks from residue and reference standard should be within $\pm$ 25%. Chromatograph reference material immediately after sample.

b. Measure PCB residues by comparing total area or height of residue peaks to total area or height of peaks from appropriate Aroclor reference material. Measure total area or height response from common baseline under all peaks. Use only those peaks from sample that can be attributed to chlorobiphenyls. These peaks must also be present in chromatogram of reference material. Mixture of Aroclors may be required to provide best match of GLC patterns of sample and reference material.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

c. Alternatively, determine PCB residues by individual peak area comparisons using Aroclor reference material weight factors in the table below. Calculate each PCB peak against appropriate individual reference peak with exactly same absolute retention. Sum individual peak values to obtain total ppm PCB.

Table 29.02. Weight % factors for individual gas chromatographic peaks in Aroclor reference materials. Peaks are identified by their retention time relative to p, p′-DDE = 100 analyzed under the same conditions.

AOAC

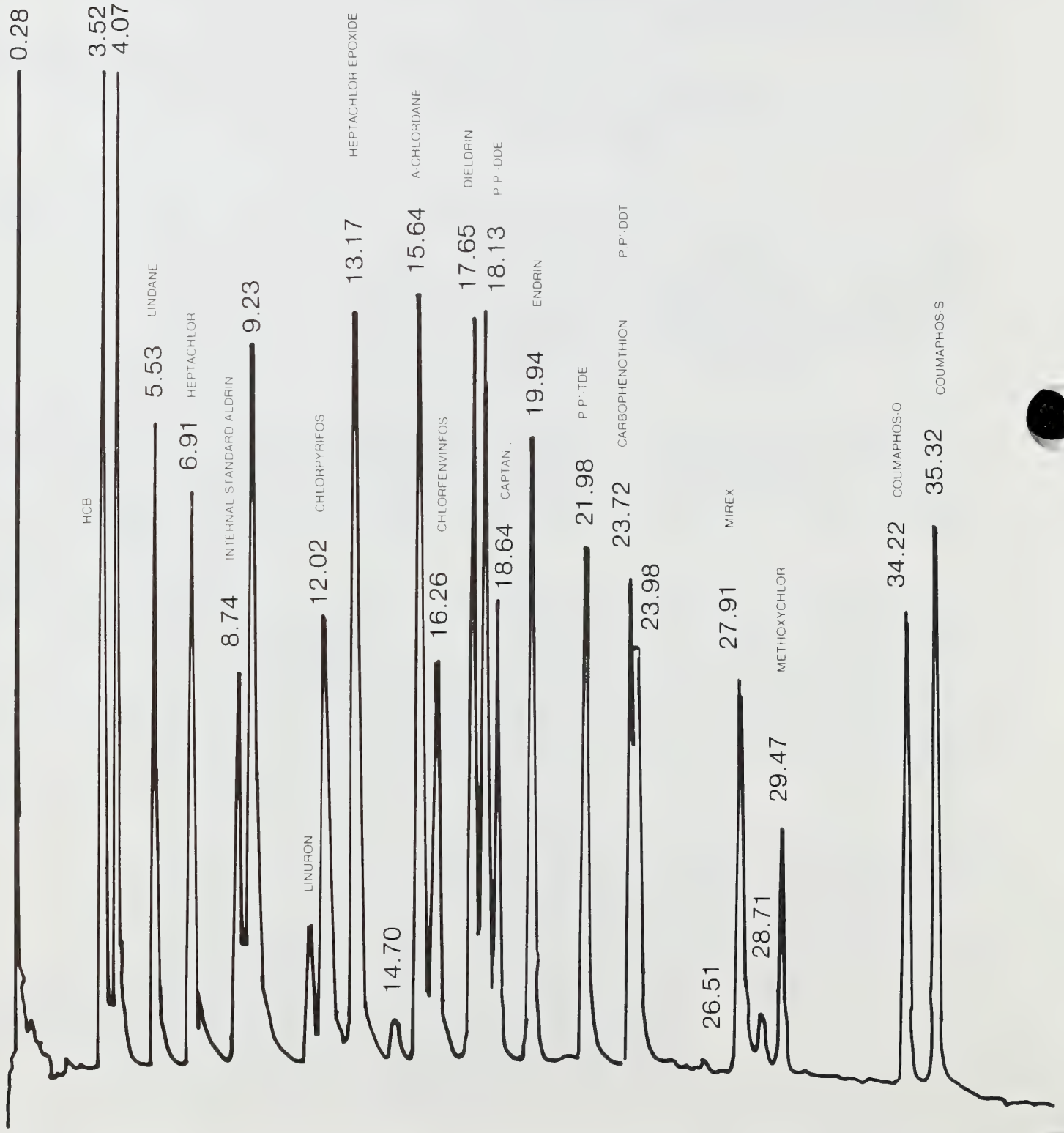
R_{DDE}(100X)	1016 (77-029)*	1242 (71-696)*	1248 (71-697)*	1254 (71-698)*	1260 (71-699)*
11	0.2				
16	3.8	3.4	0.3		
21	8.1	10.3	1.1		
24	1.2	1.1	0.2		
28	16.8	15.8	6.0		
32	7.6	7.3	2.6		
37	18.5	17.0	8.7		
40	14.6	13.0	7.4		
47	11.6	9.9	15.7	7.1	
54	7.7	7.1	9.3	2.7	
58	6.4	4.4	8.3	1.2	
70	3.4	8.7	18.2	14.7	2.4
78		1.9	6.4		
84			4.6	18.6	3.6
98			3.4	8.3	
104			3.3	14.1	2.8
112			1.0		
117					4.4
125			2.3	15.6	11.0
146			1.2	9.0	13.3
160					5.5
174				7.4	10.0
203				1.3	10.9
232-244					11.2
280					12.5
332					4.2
360-372					5.4
448					0.8
528					2.0

* Food and Drug Administration Lot Numbers. (Weight factors are valid only for these FDA Lot Numbers.) Aroclor reference materials are available from the Food and Drug Administration, Division of Chemical Technology, HFF-420, 200 C St., SW, Washington, DC 20204.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

4. Sample Chromatograms



Standard solution 0.02 μg/mL for all compounds except for linuron, coumpahos-O, and coumaphos-S (0.06 μg/mL).

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Gel Permeation Chromatography Method for Determining Organohalides in Animal Fats.

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat, shield.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
E.2. Column preparation.	The methylene chloride/ cyclohexane mixture is very flammable and can produce severe skin irritation.	Though this analysis is designed to run unattended for a greater part of the time, there is still significant potential for exposure when ''feeding'' and maintaining the GPC instrument.
E.3.a. Sample chromatography	The analyst must be fully aware of the danger of implosions during the rotary evaporation process.	The rotary evaporation system should be installed behind a shield even if this tends to make these instruments less accessible. In addition, the surrounding area should be well ventilated.

4. Disposal Procedures

Methylene chloride/ cyclohexane	See above	This mixture should be temporarily stored with the waste chlorinated solvents until disposed of by the contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Element	*Expected Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
HCB	70-120	17	20
alpha BHC	70-120	17	20
lindane	70-120	17	20
heptachlor	70-120	17	20
aldrin	70-120	17	20
ronnel	70-120	17	ND
linuron	50-120	17	20
oxychlordane	70-120	17	20
chlorpyrifos	50-120	17	20
nonachlor	70-120	17	20
heptachlor epoxide	70-120	17	20
endosulfan I	70-120	17	20
trans-chlordane	70-120	17	20
cis-chlordane	70-120	17	20
chlorfenvinphos	70-120	17	ND
dieldrin	70-120	17	20
p,p'-DDE	70-120	17	20
captan	50-120	17	ND
stirofos	50-120	17	ND
kepone	50-120	17	ND
endrin	70-120	17	20
p,p'-TDE	70-120	17	20
o,p'-DDT	70-120	17	20
endosulfan II	70-120	17	20
p,p'-DDT	70-120	17	20
carbophenothion	70-120	17	ND
mirex	70-120	17	20
methoxychlor	70-120	17	20
phosalone	70-120	17	ND
coumaphos-O	50-120	17	ND
coumpahos-S	70-120	17	ND
toxaphene	60-110	12	20
PCB 1242	80-100	12	20
PCB 1248	80-100	12	20
PCB 1254	80-100	12	20
PCB 1260	80-100	12	20

For ID only: chlordene, dichlofenthion, leptophos, beta BHC, delta BHC.

ND: not determined.

Expected recovery is determined based on the LDL listed in section J.8.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

2. Critical Control Points and Specifications	*Record*	*Acceptable Control*
	a. Chromatograms.	DB 608 or equivalent that separates for quantitation all compounds of interest (resolution). Each chromatogram to be timed for 5 minutes past the time coinciding with the retention time for the last normal eluter when screening. (This usually is coumpahos-S.) For confirmation, each chromatogram to be timed for 1 minute past the last eluting compound of interest.
	b. A solvent/tissue blank run with each GPC sample set.	No deflection $>$ 2.0% full scale from baseline from 2-60 minutes after injections of concentrated solvent.
	c. Each individual chlorinated hydrocarbon, its concentration and scale deflection, and date of test.	1-3 μL injection containing 20 pg heptachlor epoxide, giving at least a 50% scale deflection; other chlorinated hydrocarbon amounts calculated and provided in the standard solution to give about the same full-scale deflection.
	d. GPC column calibration, record of recovery data for each compound of interest. Distribution profile for each individual compound in each 10 mL fraction.	Dump-collect cycle set to obtain 70-120% or 50-100% recovery for compounds of interest, as listed under J.1.
	e. Recalibrate pressure.	Exceeds 15 psi.
	f. Rendering oven temperature.	80° C $\pm$ 3° C.
	g. Sample weight.	2.0 g $\pm$ 0.1 g fat.
	h. Correct volume of dissolved sample.	At least 8 mL of dissolved sample should be loaded in GPC sample loop.
	i. Acceptable variance of aldrin concentration.	Aldrin added is found value 70-120%.
	j. Calculations.	If the laboratory temperature varies more than $\pm$ 15° F, quantitation is accomplished using that day's recovery value. If temperature variation is $\leq$ 15° F, correction should be based on the 10-day average recovery.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—At least 3 levels, 3 replicates for each compound of interest.

(a) At minimum proficiency level.

(b) At twice minimum proficiency level.

(c) At one-half minimum proficiency level.

ii. Phase II: Fortified sample—At least 3 levels, 3 replicates each, for each compound of interest. With each set, reagent and tissue blank should also be included.

(a) At minimum proficiency level.

(b) At twice minimum proficiency level.

(c) At one-half minimum proficiency level.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Fourteen check samples including controls submitted by Science & Technology's Western Laboratory.

(b) Report analytical findings to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official sample analysis.

b. Acceptability criteria.

Refer to section J.1.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst.

ii. Blind samples (requires ''dummy'' forms) or random replicates chosen by the supervisor after initial analysis.

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) CUSUM control chart on differences between replicates.

(c) For all recoveries: the running average, standard deviation, and coefficient of variation on last acceptable 10 values.

b. Acceptability criteria.

Refer to section J.1 above.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Interlaboratory Check Sample Program

a. Laboratory responsibility.

i. Frequency: 1 every other month.

ii. Samples: Procured, prepared, and submitted by FSIS Science & Technology Western Laboratory.

iii. Report analytical findings to Chemistry Division.

b. Chemistry Division responsibility.

i. Evaluates all reported values for:

(a) Average, standard deviation, and coefficient of variation per sample set.

(b) Running average, standard deviation, and coefficient of variation for percent recoveries per sample series for last 10 samples.

(Submits written report to participating laboratories and to Program officials after all reports on each set are received.)

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

ii. Prepares ongoing control chart by laboratory code. (Submits written report to participating laboratories and to Program officials quarterly.)

iii. Phones and writes each laboratory submitting unacceptable values (cc: Director, Field Service Laboratory Division), indicating reason for unacceptability and requesting memo of acknowledgement and corrective action taken.

c. Acceptability criteria.

i. Each value reported:

(a) Outliers based on 95% confidence intervals (only if two laboratories or more are participating).

(b) Reproducibility—see section J.1.

ii. Running percent recovery—standard deviation such that coefficient of variation is ≤ 17% (for last 10 acceptable recoveries).

iii. Acceptability of analyst—not more than 1 outlier in any 3 consecutive months.

iv. Acceptability of laboratory control—no significant statistical bias.

6. Sample Acceptability and Stability

a. Matrices: Fat, extracted fat adipose tissue.

b. Sample receipt size, minimum: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests and reserve sample.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage:

i. Time: Indefinite (chlorinated hydrocarbons); estimated 1 month (organophosphates).

ii. Condition: Frozen (all compounds).

7. Sample Set

Each set processed on the same day should include:

a. Tissue blank.

b. Blank tissue fortified at tolerance level for compound of interest.

c. Sample(s).

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

8. Sensitivity

	Concentration, ppm		
Organohalides	*Lowest Detectable Level (LDL)*	*Lowest Reliable Quantitation (LRQ)*	*Minimum Proficiency Level (MPL)*
HCB	0.01	0.02	0.05
alpha BHC	0.01	0.02	0.05
lindane	0.01	0.02	0.05
heptachlor	0.01	0.02	0.05
aldrin	0.02	0.03	0.05
ronnel	0.02	0.03	0.05
linuron	0.25	0.50	0.50
oxychlordane	0.02	0.04	0.15
chlorpyrifos	0.05	0.10	0.20
nonachlor	0.03	0.06	0.15
heptachlor epoxide	0.01	0.02	0.05
endosulfan I	0.01	0.02	0.10
trans-chlordane	0.10	0.15	0.30
cis-chlordane	0.10	0.15	0.30
chlorfenvinphos	0.03	0.05	0.10
dieldrin	0.01	0.02	0.05
p,p′-DDE	0.02	0.05	0.05
captan	0.02	0.04	0.05
stirofos	0.05	0.06	0.30
kepone	0.03	0.06	0.20
endrin	0.03	0.05	0.05
p,p′-TDE	0.03	0.05	0.15
o,p′-DDT	0.04	0.05	0.15
endosulfan II	0.02	0.04	0.20
p,p′-DDT	0.03	0.05	0.15
carbophenothion	0.03	0.06	0.20
mirex	0.04	0.06	0.10
methoxychlor	0.15	0.15	0.50
phosalone	0.01	0.02	0.05
coumaphos-O	0.15	0.20	0.30
coumaphos-S	0.15	0.20	0.30
toxaphene	0.50	0.50	1.0
PCB 1242	0.30	0.30	0.5
PCB 1248	0.30	0.30	0.5
PCB 1254	0.30	0.30	0.5
PCB 1260	0.30	0.30	0.5

CHLORINATED HYDROCARBONS

Contents

Confirmatory Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Sample Preparation 5
F. Analytical Confirmation Procedure 6
G. [Reserved]
H. Hazard Analysis 8
I. Worksheet 9
J. Quality Assurance Plan 13

CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory

This is a nonvalidated confirmatory method using a capillary column for separation and electron impact ionization for detection. The method is capable of confirming chlorinated hydrocarbons at concentrations as low as 10-80% of the action level using the extract from the GPC, micro alumina column, or florisil column method. The absolute sensitivity of the confirmatory method varies from compound to compound. Confirmation generally requires injection of approximately 5% of the sample extract.

Selected ion monitoring is used to confirm the presence of the chlorinated hydrocarbons listed in A.3 below. Because six ions are monitored for each component, the suspected component and its level should be made known to the analyst to minimize time spent on the confirmatory procedure.

2. Applicability

This method is applicable for the confirmation of the following chlorinated hydrocarbons in animal fat: BHC, HCB, lindane, heptachlor, heptachlor epoxide, oxychlordane, DDE, dieldrin, endrin, TDE, DDT, methoxychlor, and mirex.

CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus	Refer to CHC3, section B.1.
2. Instrumentation	GC/MS: Hewlett Packard 5970B MSD/5890 GC equipped with 30 m DB5 column (J & W Scientific, Inc.), 0.25 mm id, 0.25 micron film thickness. 30 M OV-1 narrow bore (Hewlett Packard *Ultrabond*, part 19091A, option 105) can be used as an equivalent.

CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Refer to CHC3, section C.

b. Dry nitrogen.

c. Hexane: Burdick & Jackson, distilled in glass, or equivalent.

d. Isooctane: Burdick & Jackson, distilled in glass, or equivalent.

CONFIRMATORY METHOD

D. STANDARDS

Preparation of Standards

a. Working standards will vary for each compound depending upon its sensitivity and confirmatory concentration.

b. Each standard will be diluted appropriately to match the concentration of the sample to be confirmed.

CONFIRMATORY METHOD

E. SAMPLE PREPARATION

a. Carefully evaporate to dryness the sample extract prepared for GC injection by the acetonitrile partition/Florisil column cleanup procedure, the micro alumina column screening procedure, or the gel permeation chromatography method.

b. Dilute to 20 μL with isooctane. The volume may be varied such that injection of no more than 2.5 μL into the GC/MS produces a sufficient number of counts at each monitored ion so that confirmatory criteria can be met, using samples fortified at or below 80% of the action level.

CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

GC conditions.

i. Carrier gas: helium.

ii. Injection mode: Grob splitless.

iii. Purge flow: 25 mL/min.

iv. Purge off time: 1 min.

v. Injection temperature: 260° C.

vi. Column head pressure: 10 psi.

vii. Interface temperature: 285° C.

viii. Column temperature program (for 30M OV-1):

Temperature 1	180° C, hold 1 min
Temperature rate	16° C/min
Final temperature	250° C, hold several min

NOTE: The ''solvent elution time'' and ''run time'' are varied to create a small time window during which the CHC of interest elutes. This procedure, which is experimentally determined by the analyst/MS operator, minimizes the time during which the filament is on, thereby maximizing source cleanliness.

2. Criteria for Confirmation

a. No false negatives or positives.

b. At least four of the six ions monitored for any chlorinated hydrocarbon must be present.

c. At least two ratios, relative to the most intense ion, must be within ± 15% of those found in the blank sample fortified at the action level, or level found in the suspect sample.

d. At least one of the two ion ratios must involve ions from the highest m/z value isotopic cluster monitored, if ions in that cluster have a relative intensity > 15%.

e. The retention time must be within ± 5 seconds of that found for the standard or fortified sample.

CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

f. Ions monitored.

Compounds	*Ions Monitored/ Approximate Relative Ion Intensities*					
BHC	217 / 100	219 / 95	181 / 97	145 / 21	183 / 100	111 / ?
HCB	284 / 100	286 / 88	249 / 24	142 / 31	214 / 20	107 / ?
Lindane	183 / 100	219 / 92	217 / 83	181 / 86	146 / 20	111 / 62
Heptachlor	272 / 100	270 / 59	237 / 23	372 / 13	374 / 13	337 / 34
Heptachlor epoxide	355 / 100	353 / 80	237 / 20	263 / 13	253 / 21	272 / ?
Oxychlordane	115 / 100	387 / 69	389 / 74	310 / 34	312 / 26	424 / 11
DDE	318 / 100	316 / 76	248 / 42	246 / 92	210 / ?	176 / 14
Dieldrin	263 / 100	279 / 52	277 / 98	191 / 37	108 / 69	237 / 26
Endrin	345 / 100	347 / 58	263 / 66	245 / 56	248 / 50	173 / 40
TDE	235 / 100	237 / 68	212 / 20	178 / 31	176 / 17	165 / 30
DDT*	—	—	—	—	—	—
Methoxychlor	227 / 100	223 / 43	238 / 40	152 / 37	308 / 18	310 / 12
Mirex	272 / 100	274 / 37	270 / 48	237 / 21	235 / 18	143 / ?

* Mixture of isomers. Relative ion intensities not determined.

CONFIRMATORY METHOD

H. HAZARD ANALYSIS

Refer to CHC3, section H.

CONFIRMATORY METHOD

I. WORKSHEET

The worksheet on the following page, *Mass Spectrometry Confirmation/ Sample Information Form*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY CONFIRMATION/ SAMPLE INFORMATION FORM

(To be submitted with copy of FSIS Form 10,000-2 or equivalent)

Submitted by: _____ Date: _____

Sample I.D. Form # Lab #	UAR Approval:	Residue Code/Name 1) 2)	Violation Sample YES/NO Violation Level (ppm)

	Recovery	Tissue Blank	Sam 1	Sam 2	Sam 3	Sam 4	Sam 5
Conc. found/spiked in tissue (ppm)	1) 2)						
Absolute amount (ng) of residue in final extract (assuming 100% recovery)	1) 2)						
Tissue weight (gm) equivalent of final extract	1) 2)						

Concentration of standard submitted:

To be completed by GC/MS Operator

Final volume (μL) for SIM confirmation:

Final volume (μL) for full scan confirmation:

Approximate % recovery calculated by GC/MS:

Operator(s) _____ Start date _____

Reviewer _____ Completion date _____

CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb for all compounds.

b. No false negatives at levels listed in J.7.

2. Critical Control Points and Specifications

Refer to CHC3, section J.2.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standards, show that instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation, submitted by supervisor.

(a) A tissue blank for each type of species to be monitored.

(b) 5 samples (combination of all species), each fortified with no more than 2 types of CHCs at the actionable level.

Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to confirmation criteria, section F.2.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One check sample for confirmation on a quarterly basis.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples over a period of one year.

iii. Records are to be maintained by the analyst and reviewed by the Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on difference between replicates.

CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

If unacceptable values, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify all probable causes.

iii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Fat.

b. Sample receipt size, minimum: 50 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage:

i. Time: Indefinite.

ii. Condition: Frozen (−20° C).

6. Sample Set

Each set must include a tissue blank, a tissue blank fortified with the appropriate standard at its actionable level as well as at the highest concentration found in the suspect sample, and all samples found positive from the determinative method.

7. Sensitivity

a. Lowest detectable level (LDL): not applicable.

b. Lowest reliable confirmation (LRC):

Compound	*Lowest Reliable Confirmation (ppm)*
BHC	0.01
HCB	0.01
Lindane	0.01
Heptachlor	0.01
Heptachlor epoxide	0.10
Oxychlordane	0.10
DDE	0.02
Dieldrin	0.01
Endrin	0.03
TDE	0.04
DDT	0.04
Methoxychlor	0.15
Mirex	not available

CLOPIDOL IN LIVER AND MUSCLE TISSUES

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 7
G. Calculations 8
H. Hazard Analysis 9
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Clopidol (3,5 dichloro-2,6 dimethyl-4-pyridinol) is effective as a coccidiostat and is formulated as a 25% premix called Coyden® 25 (Dow Chemical). The premix is mixed with poultry feed to give a concentration of 0.0125% clopidol.

Poultry tissues are extracted with methanol, filtered, and an aliquot taken through cleanup procedure, employing an alumina and an anion exchange column. An aliquot is methylated in a closed system with diazomethane in a 70° C water bath. The excess reagent is evaporated, sodium hydroxide and water are added, and the mixture is extracted with benzene. An aliquot of the benzene layer containing the clopidol methyl ether (3,5-dichloro-4-methoxy-2,6-lutidine) is injected into a gas chromatograph equipped with an electron capture director. The peak height from the compound is compared to a standard curve prepared from pure methyl derivative.

NOTE: For routine screening by HPLC, see G.2.b.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Grinder.

b. Balance, top loader (Mettler P1200 or equivalent).

c. Virtis homogenizer or equivalent and accessories.

d. Filtering funnel, 60 mL, sintered glass disc, coarse (Kimax 28400 or equivalent).

e. Chromatographic column, 10 mm id × 12 cm, having a coarse fritted disc (Ace #5885-06 or equivalent).

f. Chromatographic column, 19 mm id × 16 cm, having a coarse fritted disc (Ace #5885-12 or equivalent).

g. Beakers and volumetric flasks as required.

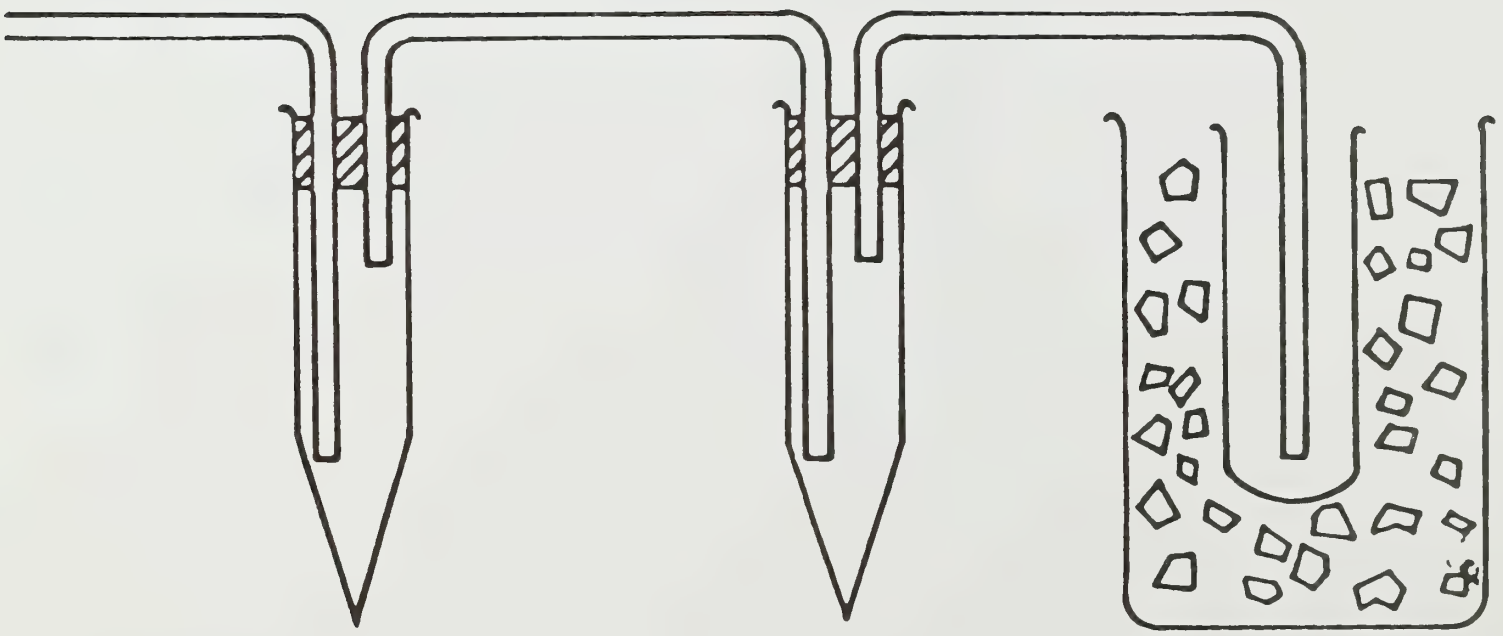
h. 70° C water bath.

i. Screw-cap culture tube with Teflon-faced cap liner (Kimax 45066-A or equivalent).

j. Micro liter syringe.

k. Diazomethane generator. Fire-polished glass tubing, 2 rubber stoppers, one 15 mL test tube, 2 plain-angle centrifuge tubes (15 mL capacity) (Kimax 45159 or equivalent).

DIAZOMETHANE GENERATOR (Fire-polish all glass tubing.)



DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

2. Instrumentation	Gas chromatograph wtih EC detector and 6 ft 3 mm id glass column packed with 25% DC-200 silicone oil or equivalent on Chromosorb W-AW 80/100 mesh (Applied Science Laboratories, State College, PA).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Alumina: Alcoa F-20, 80/100 mesh, Aluminum Company of America, East St. Louis, IL, or equivalent.

b. Anion exchange resin: AGI-X8, 100/200 mesh, acetate from Bio-Rad Laboratories, Richmond, CA, or equivalent.

c. Filter aid: Hyflo Super-Cel, Johns Manville Products Corp., New York, NY, or equivalent.

d. Methanol.

e. 5% HCl in methanol.

f. 80% Methanol in water (prepare fresh daily).

g. Carborundum chips.

h. 1M Sodium hydroxide: 4 g per 100 mL.

i. Benzene.

j. Diazomethane: Prepare as described in Pesticide Analytical Manual, Vol. II, FDA, Washington, DC, Sec. 120.227. Use the following proportions of reagents for reaction mixture: 300 mg Diazald®, n-methyl-n-nitroso-p-toluene-sulfonamide (available from Dow Chemical Co., Box 1706, Midland, MI 48640), 2.0 mL ethyl ether, 2.0 mL 60% KOH, 2.0 mL Dowanol®, carbitol 2-(2-ethoxy-ethoxy)ethanol (available from Dow Chemical Co.)

Generate diazomethane by bubbling nitrogen through reaction mixture and collect for 5 min in 10 mL ethyl ether trap submerged in ice bath. Store ethyl ether solution of diazomethane in the freezer.

CAUTION: Diazomethane is toxic, can cause specific sensitivity, and is potentially explosive. Preparations, methylations, and evaporations should be done in a hood. Avoid glass joints, etched or scratched glassware, and sharp edges. Diazomethane solutions should be stored in a freezer and should not be exposed to direct sunlight or strong artificial light. Prepare only the amount of diazomethane necessary, take precautions and use safety shields, and face shields.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Clopidol standard and 3,5-dichloro-4-methoxy 2,6-lutidine analytical standard are available from:

Dow Chemical USA
Ag-Organics Dept.
Box 1706
Midland, MI 48640

2. Preparation of Standards

a. Clopidol standard solutions (for recovery samples).

i. Solution A, 100 μg/mL: Weigh 10.0 mg clopidol, transfer to 100 mL volumetric flask. Dilute to volume with methanol.

ii. Solution B, 10 μg/mL: Dilute 10 mL solution A to 100 mL with methanol.

iii. Solution C, 1 μg/mL: Dilute 10 mL solution B to 100 mL with methanol.

b. 3,5-Dichloro-4-methoxy 2,6-lutidine standard solutions (for standard curve).

i. Solution D, clopidol equivalent 1000 μg/mL: Weigh 0.1073 g, dissolve, transfer, and dilute to volume in 100 mL volumetric flask with benzene.

ii. Solution E, clopidol equivalent 10 μg/mL: Dilute 1 mL solution D to 100 mL with benzene.

iii. Solution F, clopidol equivalent 0.2 μg/mL: Dilute 2 mL solution E to 100 mL with benzene.

iv. Solution G, clopidol equivalent 0.15 μg/mL: Dilute 1.5 mL solution F to 100 mL with benzene.

v. Solution H, clopidol equivalent 0.10 μg/mL: Dilute 25 mL solution F to 50 mL with benzene.

vi. Solution I, clopidol equivalent 0.05 μg/mL: Dilute 25 mL solution H to 50 mL with benzene.

vii. Solution J, clopidol equivalent 0.02 μg/mL: Dilute 10 mL solution H to 50 mL with benzene.

viii. Solution K, clopidol equivalent 0.01 μg/mL: Dilute 5 mL solution H to 50 mL with benzene.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

a. Weigh 20 g ground tissue (liver is the tissue of choice) into Virtis jars. Run two blank tissues with each analytical set. One is for the recovery; the other is used as a blank (G').

b. Add 2 mL solution C to one blank tissue for 0.1 ppm recovery or 1 mL solution B for 0.5 ppm recovery.

c. Add 12 g Hyflo Super-Cel to liver tissues and 2 g Hydro Super-Cel to muscle tissues.

d. Add 50 mL methanol and blend for 3 min.

e. Filter through 50 mL sintered glass funnel prepared with 2 g Hyflo Super-Cel pad using vacuum flask. Wash with methanol until about 100 mL is collected. Dilute to 100 mL.

f. Prepare alumina column with 6 g Alcoa F-20 alumina into 19 mm id × 16 cm column. Rinse with 10 mL methanol.

g. Prepare anion exchange column, using 10 mm id × 12 cm column. Slurry the AGI-8 resin with methanol. Add 1 cm resin (after settling). Rinse with 2 mL methanol.

h. Place the alumina column over the exchange resin column with collection beaker underneath, allowing flow through both columns.

i. Pipet 20 mL of sample extract onto alumina column. Wash with 10 mL methanol.

j. Remove the alumina column and replace collection beaker with 25 mL volumetric flask.

k. Elute with 2 × 10 mL portions of 5% HCl in methanol. Diliute to 25 mL with methanol.

l. In 70° C water bath, evaporate 1 mL aliquot just to dryness in screw-cap tube, using small jet of air.

m. Add 0.2 mL 80% methanol (fresh daily). Warm to dissolve residue.

n. Add 1 mL diazomethane, cap, and heat for 2 min in 70° C water bath. Remove and let sit 5 min.

o. Add small piece of carborundum and evaporate ether layer *very carefully*, just touching tip of tube to surface of 70° C water bath.

p. Add 0.1 mL 1M NaOH, 5 mL water, and 1 mL benzene.

q. Cap and shake vigorously for 1 min. Let settle and inject 3 μL benzene layer, being careful to avoid aqueous layer.

r. Determine the peak height and interpolate from the standard curve the μL/mL of clopidol represented in the benzene extract.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Preparation of Standard Curve	Condition GC column by injecting replicate aliquots of solution F until the peak height is the same for consecutive injections. To prepare the standard curve, inject 3 μL each of solutions F through K and plot peak height (mm) or area versus μg clopidol equivalent/mL.
2. Column Preparation	Condition column for 18 hours or more at 200° C with 75 to 100 mL/min nitrogen carrier gas.
3. Instrumental Settings and Conditions	a. Carrier gas: prepurified nitrogen 120 mL/min. b. Column temperature: 180° C. c. Detector temperature: 220° C. d. Injector temperature: 220° C. e. Detector operating voltage: 38 volts. f. Electrometer: Adjust for optimum response. Retention time: Approximately 4 min.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$R \text{ (recovery)} = \frac{\text{ppm clopidol found}}{\text{ppm clopidol added}}$$

$$\text{ppm clopidol} = \frac{6.25 \times (G - G')}{R}$$

$$6.25 = \text{dilution factor, i.e., } \frac{100 \text{ mL} \times 25 \text{ mL} \times 1 \text{ mL}}{20 \text{ g} \times 20 \text{ mL} \times 1 \text{ mL}}$$

G = μg/mL found in sample using the standard curve.

G′ = μg/mL found in blank using the standard curve.

2. References

a. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition.

b. Mtema, Canutte A., et al, JAOAC, No. 2, p. 334, 1984.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Clopidol in Liver and Muscle Tissue		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Benzene	Carcinogenic: This chemical has been identified as a cause of severe toxic injuries.	Used as an injection solvent for GLC. Insure that the system is vented into a fume hood.
	Diazomethane	Explosive, toxic, and potentially irritating to the skin, eyes, and respiratory system.	Refer to statement under section C.
4. Disposal Procedures	Extracted meat tissue	Solvent fumes may cause irritations.	Place waste tissues in fume hood to allow vapors to dissipate.
	Waste organic solvents	Flammable, possible toxic agents.	Gather and store in the nonchlorinated collection can until disposed of by contractor or in-house specialist.

CLORSULON IN BEEF AND SHEEP TISSUES

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 9
G. Calculations 10
H. Hazard Analysis 11
I. [Reserved]
J. Quality Assurance Plan 12

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory	The tissue sample is treated with hydroxylamine and ammonia and homogenized with ethyl acetate. The ethyl acetate is separated and removed by evaporation. The residue dissolved in an aqueous media with a small amount of acetonitrile present is first washed with hexane and then absorbed on a Bond-Elut CH column. An aliquot of the acetonitrile eluate is compared by UV-LC to a series of standards.
2. Applicability	This method is applicable to bovine kidney, muscle, and liver.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Adapters (to connect columns, etc.): Analytichem International, #636001, or equivalent.

b. Aspiration system.

c. Balance: Analytical, capable of weighing 1 mg accurately.

d. Balance: Capable of weighing 1 g and 5 g accurately.

e. Bottles: Squeeze, 50 mL.

f. Bath: Water, variable temperature 40°-80° C.

g. Bath: Ice plus water mixed in a 500 mL beaker.

h. Centrifuge: IEC Model HN-S II with six-place rotor, IEC #950 for 15 and 50 mL tubes, or equivalent system. The centrifuge is run at 2000-5000 rpm. The centrifuge used gives ca 700-750 G maximum centrifugal force.

i. Centrifuge tubes: Glass, 15 and 50 mL, with polyethylene stoppers to fit.

j. Culture tubes: Disposable, 13 × 100 mm Fisher borosilicate glass, or equivalent.

k. Columns: Bond-Elut CH (cyclohexyl)—1000 mg, 6 mL—Analytichem International, #610406, or equivalent.

l. Depth filters: Analytichem International, #650006, or equivalent.

m. Dispensing pipetters: 10 and 20 mL.

n. Filtration unit: Complete with Millipore LSWP02500 filter, or equivalent.

o. Flask: Erlenmeyer, 2 L.

p. Flask: Volumetric, 100, 500, and 1000 mL.

q. Freezer: Capable of reaching −20° C.

r. Graduates: 1 L and 250 mL.

s. Homogenizer, Polytron: Brickmann Instruments, #27-11-200-5 with PTA 10S generator, #27-11-330-3, or equivalent.

t. Meter, pH.

u. Paper: Glassine.

v. Pipets: 0.2, 1, 5, and 10 mL measuring; 0.5, 1, 5, and 25 mL volumetric and disposable.

w. Parafilm: American Can Co.

x. Refrigerator: 4°-5° C temperature.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

y. Repipet: Variable volumes.

z. Reservoirs: Analytichem International, 25 mL capacity, #602500, or equivalent.

aa. Shaker: Eberbach 6010, 2-speed, or equivalent.

bb. Spatula: Stainless steel metal.

cc. Syringe: 50 μL.

dd. Ultrasonic bath: Sonongen Automatic Cleaner, Bronson Model 520 or B32, or equivalent.

ee. Vac Elut: Analytichem International, or equivalent.

ff. Vortex mixer.

2. Instrumentation

Chromatographic apparatus: Beckman-Altex model 110A liquid chromatographic pump complete with Valco sample valve with syringe-loading sample loop or Waters Wisp Autosampler and Kratos-Schoffel Instruments Spectroflow Model 773 UV absorbance detector with a 110 millivolt recorder, or equivalent, is used. A 5 μm, 4.6 mm id C_{18} standard BrownLee Labs guard column (Spheri-5 RP-18 OD-GU obtained from Rainin Instrument Co., Inc., or equivalent) is used before the analytical column. This guard column is replaced frequently, whenever pressure buildup and/or deterioration of peak is observed.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Acetonitrile: Burdick and Jackson, or equivalent.

b. Ammonium hydroxide: Concentrated or 29%.

c. Ethyl acetate: Pesticide grade.

d. Hexane: Burdick and Jackson, or equivalent.

e. Hydrochloric acid: 1M.

f. Hydroxylamine hydrochloride: ACS reagent. (If the hydroxylamine hydrochloride is not finely ground, it should be ground in a mortar and pestle.)

g. Sodium bisulfite.

h. Sodium hydroxide: 50% (w/w) analytical reagent.

i. Potassium phosphate: Monobasic, anhydrous; ACS reagent.

j. Water: Suitable for HPLC, such as provided by a Millipore Milli-Q TM system.

k. Hydrochloric acid: 0.002M. Dilute 1 mL of the 1M HCl to 500 mL with distilled water.

l. Buffer: 0.01M. Weigh 1.36 of the potassium phosphate into a 2 L Erlenmeyer flask. Add 1 L of Millipore water, or equivalent, and stir to completely dissolve. Use a pH meter to adjust the pH to 7.0 with 50% (w/w) sodium hydroxide. Filter through a Millipore LSWP02500 filter, or equivalent, using vacuum. Make fresh at least weekly.

m. Mobile phase: Mix 800 mL of the 0.01M pH 7.0 buffer with 200 mL acetonitrile. Sonicate in an ultrasonic bath for 30 min to degas.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Merck, Sharp & Dohme Research Laboratories
Division of Merck, Inc.
Manager, Chemical Data Dept.
P.O. Box 2000
Rahway, NJ 07065-0900
Telephone: (201) 594-5744

2. Preparation of Standards

a. Stock standard (0.5 mg/mL): Weigh 50 mg of clorsulon into a 100 mL volumetric flask and dilute to volume with 50% methanol/water.

b. Fortification standard (0.10 mg/mL): Add 10 mL of the stock standard to a 50 mL volumetric flask and dilute to volume with water.

c. Working standards: Prepare as follows.

Fortification Standard (mL used)	*Final Volume (mL)*	*Final Concentration (ppm)*
0.5	10	5.0
1.0	10	10.0
2.0	10	20.0

3. Storage Conditions

All standards are to be refrigerated at 4° C when not in use. Warm to room temperature before each use.

4. Shelf Life Stability

Stability is based upon the storage conditions listed in section D.3.

a. Stock standard: One year.

b. Fortification standard: Every two months.

c. Working standards: Every two months.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Homogenization and Extraction

a. Weigh onto a series of pieces of glassine paper 1 g of solid hydroxylamine hydrochloride for each sample to be run.

b. Into each of a series of 50 mL centrifuge tubes, add 20 mL ethyl acetate by dispensing pipetter. Prepare one tube for each sample to be run (tube 2).

c. Weigh accurately 5 g of tissue into a 50 mL centrifuge tube (tube 1).

d. Weigh accurately 5 g of blank tissue. Add 50 μL of fortification standard to yield 1.0 ppm recovery standard.

e. To each individual tissue sample as given add in rapid order:

   i. One of the weighed solid hydroxylamine samples, added in one spot down one side of the tube, on top of the tissue.

   ii. Any fortification in water in a small volume (0-0.2 mL). The fortification should be carefully added on top of the hydroxylamine.

   iii. 0.1 mL of 29% ammonia by graduated pipet.

   iv. 20 mL of ethyl acetate by dispensing pipetter.

f. With the tube surrounded by an ice water bath, homogenize the tissue/ethyl acetate mix (tube 1) at a setting of 6-7 until the meat and mix are thoroughly homogenized. A 15-20 sec homogenization is usually sufficient and the time should not exceed 30 sec. Do step e and f for one sample at a time.

g. Scrape off any loose pieces of tissue into the centrifuge tube using a spatula and rehomogenize briefly with the corresponding tube 2 (5-10 sec is sufficient). [The second homogenization with clean solvent is intended to get any clorsulon left on the probe from the first homogenization.] Between the tube 2 rinse and the next sample, the probe should be cleaned by sequentially homogenizing briefly with methanol, water, and ethyl acetate and wiping off with a clean paper towel.

h. Cap tube 1 and shake for 30 min on low speed on a shaking machine with tubes lying flat and the length of the tube oriented in the direction of shaking.

i. Centrifuge tube 1 at full speed for 5 min and decant the clear supernatant into a third 50 mL tube (tube 3).

j. Stopper and shake the corresponding tube 2.

k. While still shaking tube 2, pour its contents into tube 1 and use a vortex mixer to thoroughly break up the tissue plug.

l. Stopper and reshake tube 1 as before for 10 min.

m. Again centrifuge for 5 min and again decant the supernatant, combining the ethyl acetate phases in tube 3.

n. Evaporate to an oil that strongly resists further evaporation, using a nitrogen flush and a water bath at 60°-70° C.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Hexane/Water-Acetonitrile Wash

a. While the oil is still warm and molten, add by dispensing pipetter 10 mL of hexane and swirl to dissolve.

b. Add, by 5 or 10 mL pipet, 1 mL of acetonitrile. Stopper and use ultrasound to dissolve any clorsulon residue in the acetonitrile. Shake at low speed for a minute on a mechanical shaker.

c. Add by pipet exactly 25 mL of 0.002M hydrochloric acid and shake at low speed for a minute on a mechanical shaker.

d. Centrifuge for 10 min. (The upper phase and interface can be aspirated to waste at this time if the analyst feels such aspiration is helpful for the subsequent step. If aspiration is used, leave plenty of the lower phase for the subsequent 20 mL pipetting.)

3. Variation for Fat

Treat sample exactly the same as for other tissues except that:

a. The centrifugations of the ethyl acetate homogenate should be for 10 min each.

b. The first homogenization should be with 30 mL of ethyl acetate.

c. The decantations should be through a glasswool plug on a funnel as a filter.

d. After the first two ethyl acetate extracts are about half-evaporated, a third 20 mL ethyl acetate extract should be made and combined with the partially evaporated initial two extracts. After the third extract passes through the glass wool, squeeze out the liquid in the glass wool with a spatula into the centrifuge tube collector. The residue from the fat/ethyl acetate extractions should be 4-5 mL of oil.

4. Preparation of Bond-Elut Column System

Prepare Bond-Elut columns just before use as follows:

a. Rinse with one column volume of methanol (using methanol from a squeeze bottle).

b. Rinse with twice one column volume (6 mL) of distilled water using a squeeze bottle. Rinsing is accomplished by placing the column on the vacuum manifold (Vac-Elut), adding solvent (methanol or water), and then applying low vacuum to slowly suck solvent through the column (pressure = 10″ Hg). The vacuum can be adjusted using the needle valve on the manifold or the main valve. Release the vacuum slowly and carefully between applications of solvent.

c. Rinse depth filters twice with about 6 mL of water and partially dry by sucking air through them for a minute. (The drying period should not be unduly extended or the column will be too dry to absorb the clorsulon.)

d. Assemble a clean reservoir on top of a rinsed depth filter on top of a rinsed CH Bond-Elut column and place on the Vac-Elut, connecting each part with the blue adaptors. (If a Vac-Elut is not available, individual column setups can be prepared and used by attaching to a vacuum flask, using a one-holed stopper.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

5. Bond-Elut Chromatography

a. Prepare Bond-Elut column setups as in section E.4 and pipet exactly 20 mL of the essentially clear bottom phase (avoiding solids) into the reservoir of a Bond-Elut-CH column setup without vacuum.

b. Carefully apply vacuum to load the sample onto the column slowly, eventually increasing the vacuum to about 10″ of Hg. The column effluent looks very much like the sample applied. If the filter plugs with solids, the filter surface can be gently scraped using a disposable pipet to resuspend the solids.

c. After all the solution has passed through the system, rinse the reservoir twice with about 5 mL each of distilled water from a squeeze bottle, continuing the vacuum. The first wash should pass through the system completely before the second is started.

d. Remove the reservoir and repeat the washing of the filter. Continue washings until the eluate is clear and essentially colorless. One or two washings is usually sufficient.

e. Remove the filter and rinse the column with about 3 mL more of water.

f. Dry the column by continuing to pull air through after apparent dryness for up to about 1 min. (This is an acceptable stopping point. Store dried extract in refrigerator for no more than 3 days.)

g. Slowly release the vacuum and add to the column by repipet 4 mL of acetonitrile.

h. Put a collecting 13 × 100 mL test tube in place and slowly reapply the vacuum again up to 10″ of Hg, making sure that all of the acetonitrile has been eluted before the vacuum is again slowly released. *Do not* remove the columns from the system while the vacuum is still on or apply too much vacuum, or splashing will occur.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Liquid Chromatography

a. Evaporate the acetonitrile using a nitrogen purge and 60°-70° C bath. The purge should be increased gradually to avoid spattering as the volume goes down. The fully dried sample generally should have a small amount of scum left and no liquid.

b. Add exactly 0.5 mL of mobile phase and dissolve using ultrasound and vortex mixing. A 30-sec ultrasound treatment is sufficient. Larger dissolution volumes can be used for relatively high expected results. (Sample may be stored overnight in refrigerator.)

c. Thoroughly mix and run LC of 40 μL against 40 μL each of a series of standards. If the LC determination is to be delayed, store in a refrigerator at 4°-5° C or for longer periods in a freezer at −20° C. Firmly cover with parafilm during storage.

2. Instrumental Settings and Conditions

a.	Mobile phase	Acetonitrile/pH7, 0.01M phosphate (20/80).
b.	Column temperature	32° C.
c.	Flow	2.0 mL/min (usual pressure 1000 psi, 500-2500 psi acceptable).
d.	Retention time	ca 18 min.
e.	Wavelength	265 nm.
f.	Absorbance scale	Variable down to 0.005-0.006 AUF.
g.	Calculations	By peak height, manually.

In general, the effectiveness of the LC system is judged by running clorsulon standards. If the peak height, quality of the curve, tailing, etc., have deteriorated, the precolumn is replaced or the LC further investigated.

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Plot a graph of peak height vs μg/mL of standard. The curve should be linear and go through the origin.

b. Calculate ppm = μg/mL $\left[\frac{(V_1)(V_2)}{(V_3)(W)}\right]$ = $(1.3)(\mu g/mL)(\frac{V_1}{W})$

where μg/mL for the unknown is read from the standard curve.

V_1 = mL sample dissolved for LC.

V_2 = mL sample dissolved before Bond-Elut = 26.

V_3 = mL aliquot used for Bond-Elut = 20.

W = grams of sample weighed.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Analysis of Clorsulon in Beef and Sheep Tissues

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Acetonitrile Hexane Methanol	These solvents are all flammable and may produce toxic effects to the skin, eyes, and respiratory system. Vapors are hazardous.	Work in efficient fume hood, away from electric heaters. Use plastic gloves.
Ammonium hydroxide	Very corrosive. Ingestion will result in vomiting, collapse.	Same as above.

4. Disposal Procedure

Organic solvents	See above	Dispose of in proper container.
Ammonium hydroxide	See above	Flush down sink with lots of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery %*	*Repeatability %CV*
Clorsulon	≥ 0.5	75-105	≤ 12

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5 g ± 0.1 g.
b. Acetonitrile volume	1.00 mL ± 0.01 mL.
c. 0.002M HCl solution volume	25 mL ± 0.1 mL.
d. Bond-Elut column preparation	Do not allow the Bond-Elut column to dry excessively.
e. Aliquot size from acetonitrile/HCl bottom phase	20 mL ± 0.1 mL.
f. Final volume	500 μL ± 10 μL.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each, on 3 separate days.

(a) Blank.

(b) 5 μg/mL.

(c) 10 μg/mL.

(d) 20 μg/mL.

ii. Phase II: Fortified samples—4 levels, 3 replicates each over a minimum of 3 days.

(a) Blank.

(b) 0.5 ppm.

(c) 1.0 ppm.

(d) 2.0 ppm.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Check samples for analyst accreditation.

(a) 14 samples from Western Laboratory (samples submitted by supervisor if only one laboratory is performing this test).

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

Refer to section J. 1 above.

4. Intralaboratory Check Samples

a. System—minimum contents.

i. Frequency: 2 per month per analyst, or 20% of official samples analyzed (whichever is smaller).

ii. Blind samples (requires "dummy" forms).

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All % recoveries.

(d) Running average, standard deviation, and CV for recoveries.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Bovine kidney.

b. Sample receipt size: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests.

c. Condition upon receipt: Frozen.

d. Sample storage: Not established.

6. Sample Set

a. Tissue blank.

b. Tissue blank fortified at 0.5 ppm.

c. Samples.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

7. Sensitivity	a. Lowest detectable level (LDL): 0.25 ppm. b. Lowest reliable quantitation (LRQ): 0.50 ppm. c. Minimum proficiency level (MPL): Not established.

CRESYLIC ACID IN FAT

Contents

A. Introduction 1
B. Equipment 2
C. Reagents 3
D. [Reserved]
E. Extraction Procedure 4
F. Analytical Quantitation 5
G. [Reserved]
H. Hazard Analysis 6
I. [Reserved]
J. Quality Assurance Plan 7

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory	Cresylic acid is used in poultry husbandry as an employee's foot bath to prevent the shoes from becoming a vector for disease. It has the potential to become a contaminant in poultry because of its solubility in fat. Cresylic acid is also slightly soluble in water and is difficult to extract from fat or water with organic solvents. The boiling point and fat solubility of cresylic acid are high enough that adipose tissue can be safely rendered at 100° C with no significant loss. The rendered fat is dissolved in hexane, extracted with 5N sodium hydroxide, derivatized with dichloroacetylchloride, extracted with chloroform, and, after separation and evaporation of the chloroform, dissolved in hexane and analyzed by GC-EC.
2. Applicability	This method is applicable to poultry fat.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus	a. Scintillation vial, 20 mL, Wheaton Scientific No. 98654, or equivalent (with teflon-lined cap). b. Glass-stoppered 50 mL centrifuge tube, Corning No. 8424, or equivalent, with #13 standard taper ground glass stopper. c. Serological pipets, disposable, 9", Scientific Products, #P5211-2, or equivalent. d. Centrifuge, Damon/IEC HN-SII, or equivalent. e. Glass fiber filter, 9.0 cm circles, Reeve Angel 934AH, or equivalent.
2. Instrumentation	Gas chromatograph, Hewlett-Packard 5710A (or equivalent), equipped with electron capture detector (NI^{63}).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent List

a. Hexane, UV grade, Burdick and Jackson, or equivalent.

b. Chloroform, pesticide grade, Omnisolv CX1059-1, or equivalent.

c. Dichloroacetylchloride, Eastman, redistilled, or equivalent.

d. 5N NaOH, reagent grade, Fisher Scientific, SO-S-256, or equivalent.

e. Toluene, pesticide grade, Burdick and Jackson, or equivalent.

f. Sodium sulfate anhydrous, analytical grade, Mallinckrodt, or equivalent.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation	A sample of adipose tissue is rendered at 100° C, filtered through glass fiber filter, and weighed into a 50 mL centrifuge tube.
2. Sample Extraction	a. Weigh 4.0 g ± 0.01 g rendered fat into a 50 mL centrifuge tube and add 10 mL hexane. Stopper and shake until fat is dissolved. b. Add 1.0 mL 5N NaOH and gently shake for 2 minutes. c. Add 4.0 mL distilled H_2O and gently shake for 1 minute. d. Centrifuge at 1500 rpm for 2 minutes and aspirate the organic (hexane) layer. e. Add 10 mL hexane to the aqueous phase and mix layers by rotating tube end over end (bicycling) for 10 seconds. f. Centrifuge at 1500 rpm for 30 seconds and aspirate the organic (hexane) layer. g. Add 5.0 mL chloroform and 1 drop of dichloroacetylchloride (DCA) to the aqueous phase and shake vigorously for 1 minute. h. Add 1 more drop of DCA and shake vigorously for 30 seconds. i. Centrifuge at 1500 rpm for 2 minutes and aspirate the aqueous layer. j. Add 2.0 g to 3.0 g anhydrous sodium sulfate to the organic phase and mix. k. Transfer organic chloroform layer to a 20 mL scintillation vial via a disposable pipet, add 1.0 mL toluene, and evaporate to approximately 200 μL final volume.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumenal Settings and Conditions	a. Column: 6 ft × 2 mm glass column packed with 20% OV-225/ 20% OV-17 (1 + 1) on 80/100 mesh Chromosorb W-HP. b. Carrier gas: Argon/Methane 95:5 at 50 mL/min. c. Temperature: i. Isothermal—210° C. ii. Injector—250° C. iii. Detector—350° C.
2. General Operation	Add 2.0 mL hexane to the final volume from E.2.k and inject 3.0 μL on the gas chromatograph. This will produce a retention time for the cresylic acid dichloroacetate ester of approximately 5 minutes.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination of Cresylic Acid in Fat

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Hexane Chloroform	Corrosive and flammable. Vapors can cause severe skin, eye, and respiratory irritation.	Work should be performed in an efficient fume hood away from heat-generating devices.
5N NaOH	Caustic. Can cause irreversible skin damage.	Wear the required protective equipment. Be constantly aware of the chances of spattering when performing a given step in the procedure.

4. Disposal Procedures

Organic solvent mixtures	See above	Reduce volume by partial evaporation in a fume hood. Segregate chlorinated from nonchlorinated solvents before disposal by the contract or in-house specialist.
NaOH waste	See above	Dilute and flush into waste sink with large amounts of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Element	*Acceptable Recovery %*	*Analytical Range (ppm)*	*Repeatability %CV†*
Cresylic acid	40-60	0.5-2.0	—‡

† Analytical procedure is for detection only. Quantitative estimates may be determined with usual repeatability ≤ 8%.

‡ Not established.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Rendering oven temperature	100° C ± 1° C.
b. Weight of sample	4.00 ± 0.01 g.
c. Aspiration (E.2.d)	Leave small amount of organic layer.
d. Aspiration (E.2.f)	Completely remove organic layer.
e. Evaporation	Remove excess DCA.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—3 levels, 3 replicates each: 0.5 ppm (0.4 μg/mL), 1.0 ppm (1.0 μg/mL), and 2.0 ppm (2.0 μg/mL).

ii. Phase II: Fortified samples—3 levels, 3 replicates each plus tissue blanks over a minimum of 3 days: 0, 0.5, 1.0, and 2.0 ppm.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Check samples for analyst accreditation.

(a) 14 samples from FSIS Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report analytical findings to Chemistry Division.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

i. Coefficient of variation of ≤ 10% for recoveries, mean ratios.

ii. Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst, or 20% of official samples analyzed (whichever is smaller).

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

ii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All % recoveries.

(d) Running average, standard deviation, and CV for recoveries.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

i. Coefficient of variation for running average on replicate analyses of $< 20\%$.

ii. Coefficient of variation for running average of the last 10 successful recoveries of $< 20\%$, with all recoveries in range stated in section J.1.

iii. If unacceptable values are obtained, then:

(a) Stop all official analyses for that analyst.

(b) Investigate and identify probable cause.

(c) Take corrective action.

(d) Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Poultry fat.

b. Sample receipt size: Varied; enough to produce matrix for all required analyses.

c. Condition upon receipt: Frozen and enclosed in an air-tight glass or metal container.

d. Sample storage:

i. Time: Analyte is volatile (b.p. about 200° C). Should be stable if stored as stated in d.ii.

ii. Condition: Rendered and frozen; in tightly sealed glass vials.

6. Sensitivity

a. Lowest detectable level (LDL): 0.5 ppm.

b. Lowest reliable quantitation (LRQ): 0.5 ppm.

c. Minimum proficiency level: Not established.

CYANIDE IN TISSUE, PROCESSED MEATS, AND SERUM

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. [Reserved]
G. [Reserved]
H. [Reserved]
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Cyanide ion reacts with p-Nitrobenzaldehyde to form an active reductant, a cyanohydrin, which reacts with o-dinitrobenzene to form a highly colored purple compound. The reaction is specific both for p-nitro or p-cyano benzaldehyde and the cyanide ion. Cyanide ion is regenerated in the second reaction and thus acts as a catalyst, increasing the sensitivity of the method. The detection limit for cyanide ion is approximately 0.5 ppm.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

a. Culture tubes, borosilicate, 10 × 75 mm, Scientific Products, T-1285-2, or equivalent.

b. Centrifuge tube, screw-cap, 15 mL, Corning 25310, or equivalent.

c. Volumetric flask, 100 mL, Kimax 28013, or equivalent.

d. Centrifuge, Damon/IEC, clinical, or equivalent.

e. Tissuemizer, Tekmar model SDT, shaft number 10N, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. p-Nitrobenzaldehyde, Sigma N-6126, or equivalent, 0.1M in methyl cellosolve (ethylene glycol monomethyl ether).

b. o-Dinitrobenzene, Sigma D-2638, or equivalent, 0.1M in methyl cellosolve (ethylene glycol monomethyl ether).

c. Methyl cellosovle (ethylene glycol monomethyl ether), Sigma E-5378, or equivalent.

CAUTION: Toxic, combustible, harmful if inhaled or absorbed through the skin.

d. Sodium hydroxide, reagent grade, 0.5M in H_2O, Sigma 925-30, or equivalent.

DETERMINATIVE METHOD

D. STANDARDS

1. Source	Potassium cyanide, reagent grade, Fisher Scientific Company, P-226, or equivalent.
2. Preparation of Standards	a. Stock solution, 100 mg CN/100 mL. b. Reference solution A, 0.05 mg CN/100 mL. c. Reference solution B, 0.25 mg CN/100 mL.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 1.0 g of serum into a 15 mL screw-top disposable centrifuge tube. The sample should be weighed with minimum contact with laboratory equipment, especially metals, and disposable or *clean* labware is imperative to avoid extraneous color development. Add 5 mL 0.1M p-nitrobenzaldehyde.

NOTE: If serum cannot be separated from the tissue sample furnished or if processed meat is to be analyzed, homogenize 2.5 g of lean tissue or 3.0 g of processed meat with 5.0 mL H_2O and weigh 1.0 g of resulting fluid into the centrifuge tube, as for serum.

b. Shake tube for 2 min and add 5.0 mL 0.1M o-dinitrobenzene.

c. Shake tube for 2 min and and centrifuge for 5 min at approximately 3000 rpm.

d. Transfer 1.0 mL of supernatant liquid to a 10 × 75 mm culture tube using a disposable pipet, carefully avoiding any solid material. (If solid material cannot be avoided, filter supernatant using a fast paper such as Reeve Angel 802, or equivalent.)

e. Add 0.2 mL 0.5M NaOH. Mix by holding the tube with the thumb and index finger of one hand and flicking the tube with the index finger of the other hand.

f. Observe the tube for 1 min. Note any purple color. Place in the dark for 15 min and again examine for purple color.

g. If any color other than purple develops, such as brown or yellow brown, repeat the analysis using labware that is known to be clean.

h. If any other color develops, or other color develops in repeated analysis, submit the sample for confirmation by GC/MS.

2. Quality Control

Each time the analysis is performed, the following control samples should be analyzed:

a. Reagent blank (1.0 mL H_2O, 5 mL 0.1M p-nitrobenzaldehyde, 5 mL 0.1M o-dinitrobenzene, and 0.2 mL 0.5M NaOH).

b. Tissue blank (1.0 g of serum or slurry from blank sample, 5 mL 0.1M p-nitrobenzaldehyde, 5 mL 0.1M o-dinitrobenzene, and 0.2 mL 0.5M NaOH).

c. 0.5 ppm fortified standard (1.0 mL reference solution A, 1.0 g of serum or slurry from blank sample, 5.0 mL 0.1M p-nitrobenzaldehyde, 5.0 mL 0.1M o-dinitrobenzene, and 0.2 mL 0.5M NaOH).

d. 2.5 ppm fortified standard (1.0 mL reference solution B, 1.0 g of serum or slurry from blank sample, 5.0 mL 0.1M p-nitrobenzaldehyde, 5.0 mL 0.1M o-dinitrobenzene, and 0.2 mL 0.5M NaOH).

e. If no color develops in 2.c or 2.d, analyze a fortified reagent standard: 1.0 mL H_2O, 5.0 mL 0.1M p-nitrobenzaldehyde, 5.0 mL 0.1M o-dinitrobenzene, and 0.2 mL 0.5M NaOH).

DIBUTYLTIN

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 6
F. Analytical Quantitation 9
G. Calculations 13
H. Hazard Analysis 14
I. Worksheet 15
J. Quality Assurance Plan 19

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Organotin compounds have been utilized for many years to treat hexametiasis and as anthelmintic drugs for the control of parasitic worms in turkeys. Due to the potential toxicological properties of organotin, a need arose for a procedure that would be able to differentiate between organic and inorganic tin in a biological tissue matrix.

Organotin is extracted from the tissue using ethyl acetate, followed by a clean-up through a sep-pak silica column. The extract can be quantitatively confirmed using HPLC equipped with a post column derivatization unit and a fluorescence spectrophotometric detector.

2. Applicability

This method has been evaluated for organotin analysis in turkey liver and muscle.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: An equivalent can be substituted if necessary for all apparatus and equipment listed below.

a. Reciprocating shaker (Eberbach #6010).

b. Sorvall centrifuge (#T6008).

c. Rinco rotary vacuum evaporator.

d. Sep-pak silica cartridges (Waters, P/N 51900).

e. Connecting tee for joining three fluid lines (Rainin P/N U-429).

2. Instrumentation

a. HPLC (Waters, model #2010 equipped with a fluorescence detector, model #420).

b. HPLC pump (Varian, model #2010).

c. Analytical column: Adsorbosphere CN column, 150 mm × 4.6 mm; 3-5 μ particle size (Alltech Associates, Inc., P/N 287203).

d. Guard column: CN, 3-5 μ particle size.

e. Pulse dampener constructed from an approximately 10 m length of 0.01 inch ID tubing.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

HPLC grade is required for all organic solvents listed below.

a. Saline solution (8.5 g/L).

b. Hydrochloric acid, conc.

c. Acetic acid, conc.

d. Ethyl acetate.

e. Hexane.

f. Morin (Sigma Chemical #M-4008): 0.005% solution; weigh 50 mg in a clean 1000 mL volumetric flask and dilute to volume with ethanol.

g. Mobile phase: hexane:ethyl acetate (95:5) containing 3% acetic acid; add 50 mL ethyl acetate in a clean 1000 mL volumetric flask, add 30 mL acetic acid, and dilute to volume with hexane.

h. Sodium chloride.

i. Ethanol.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Dibutyltin dilaurate (DBTDL)	Pfaltz and Bauer Co. 172 E. Aurora Street Waterbury, CT 06708	Lot #Di2470 98%
b. Di-n-octyltin dilaurate (DOTDL) [Internal Standard]	Pfaltz and Bauer Co.	Lot #D48690

2. Preparation of Standards

a. Stock standard—1000 μg/mL

i. DBTDL—Weigh 25 mg and quantitatively transfer to 25 mL volumetric flask and dilute to volume with hexane.

ii. DOTDL—Weight 25 mg and quantitatively transfer to 25 mL volumetric flask and dilute to volume with hexane.

b. Working standards—250 μg/mL

i. DBTDL—Pipet 2.5 mL of stock standard into 10 mL volumetric flask and dilute to volume with hexane.

ii. DOTDL—Pipet 2.5 mL of stock standard into 10 mL volumetric flask and dilute to volume with hexane.

c. Fortification standard solution:

i. Solution 1: 12.5 μg/mL DBTDL, 25.0 μg/mL DOTDL. Pipet 0.5 mL of DBTDL and 1.0 mL of DOTDL to a 10 mL volumetric flask. Dilute to volume with ethanol.

ii. Solution 2: 25.0 μg/mL DBTDL, 25.0 μg/mL DOTDL. Pipet 1.0 mL of DBTDL and 1.0 mL of DOTDL to a 10 mL volumetric flask. Dilute to volume with ethanol.

iii. Solution 3: 50.0 μg/mL DBTDL, 25.0 μg/mL DOTDL. Pipet 2.0 mL of DBTDL and 1.0 mL of DOTDL to a 10 mL volumetric flask. Dilute to volume with ethanol.

d. Internal standard solution for samples—Pipet 1.0 mL of 250 μg/mL DOTOL into a 10 mL volumetric flask and dilute to volume with ethanol (25 μg/mL).

e. LC standards.

i. From working standards pipet 50, 100, and 200 μL of DBTOL into individual 10 mL volumetric flasks and add 100 μL of DOTDL to each flask. Dilute to volume with hexane (1.25 μg/mL, 2.5 μg/mL, 5.0 μg/mL—equivalent to 0.25, 0.50, and 1.00 ppm).

ii. From fortification standards pipet 1 mL into individual 10 mL volumetric flasks and dilute to volume with hexane. Concentrations same as above.

DETERMINATIVE METHOD

D. STANDARDS (Continued)

3. Storage Conditions	All standards are to be kept in a tightly closed glass bottle at room temperature.
4. Shelf Life Stability	a. Stock standard solution: One month. b. Intermediate standard solution: Two weeks. c. HPLC standard solution: One week. d. Fortification standard solution: One week.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Fortification

Weigh 5 g of blank tissue and fortify with 100 μL of 25 μg/mL fortification solution. This will constitute an 0.5 ppm recovery sample.

2. Sample Extraction

a. Weigh 5.0 g of prepared turkey tissue into a 50 mL polypropylene centrifuge tube. Add 10 mL saline solution and shake for 10 seconds to mix well.

b. Slowly add 8 mL concentrated hydrochloric acid, mix thoroughly, and allow to stand for 5 minutes.

c. Add 3 g sodium chloride and 20 mL ethyl acetate and shake vigorously by hand for 5 seconds.

d. Place the polypropylene centrifuge tube into a mechanical shaker and shake on high speed for 10 minutes.

e. Centrifuge at 3000 rpm for 5 minutes. Transfer the supernatant into a 100 mL round bottom flask with a disposable pipet (an automatic pipet filler may be used at this step).

f. Repeat steps c, d, and e.

g. Place the round bottom evaporation flask on a rotary evaporator and concentrate the sample extract, under reduced pressure, at a water bath temperature of 35° C to a volume of 0.5-1.0 mL. (Do not let go to dryness).

h. Quantitatively transfer the residue from the round bottom flask to a 15 mL centrifuge tube with 9 mL hexane (final volume of about 10 mL). Stopper and mix for about 10 seconds.

i. Place the centrifuge tubes, unstoppered, in a refrigerated centrifuge (at 0 to −10° C) and centrifuge for 10 minutes and 1500 rpm.

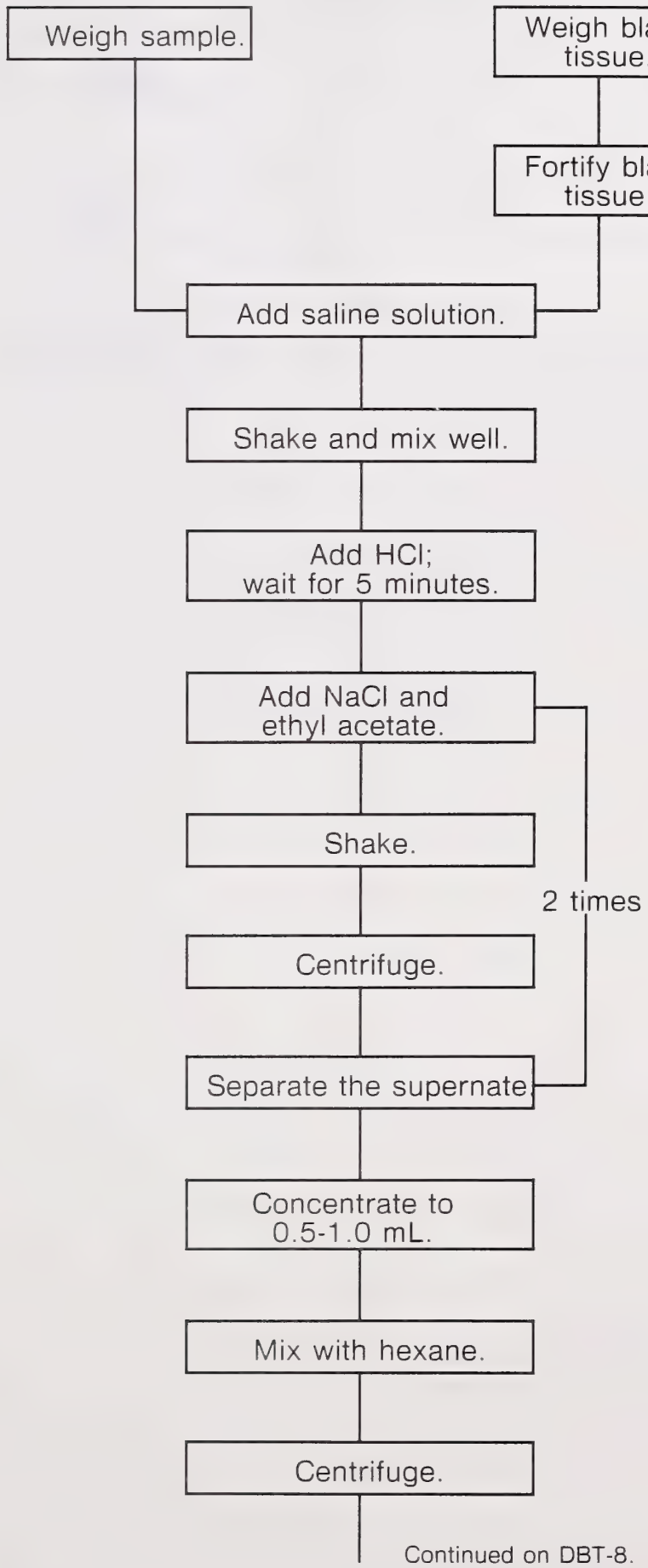
j. Transfer the hexane layer, through a prewashed silica sep-pak (prewashed with about 3 mL hexane), into a clean 15 mL centrifuge tube. (A 10 mL syringe can be used to push the sample, with gentle pressure, through the sep-pak.) Wash the sep-pak with 5 mL hexane. The total volume of the extract is about 15 mL.

k. Evaporate in a N-Evap to a final volume of 1 mL under nitrogen at 35° C. Mix thoroughly. The samples are now ready for analysis by HPLC.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

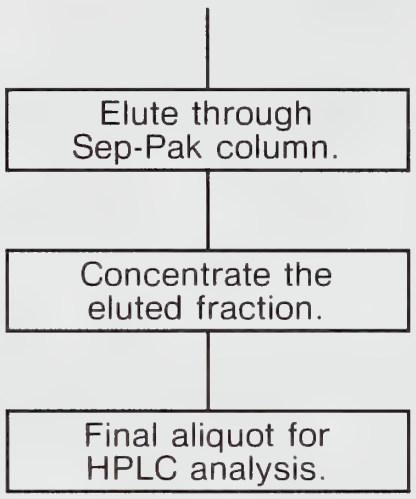
3. Flow Chart Summary



Continued on DBT-8.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

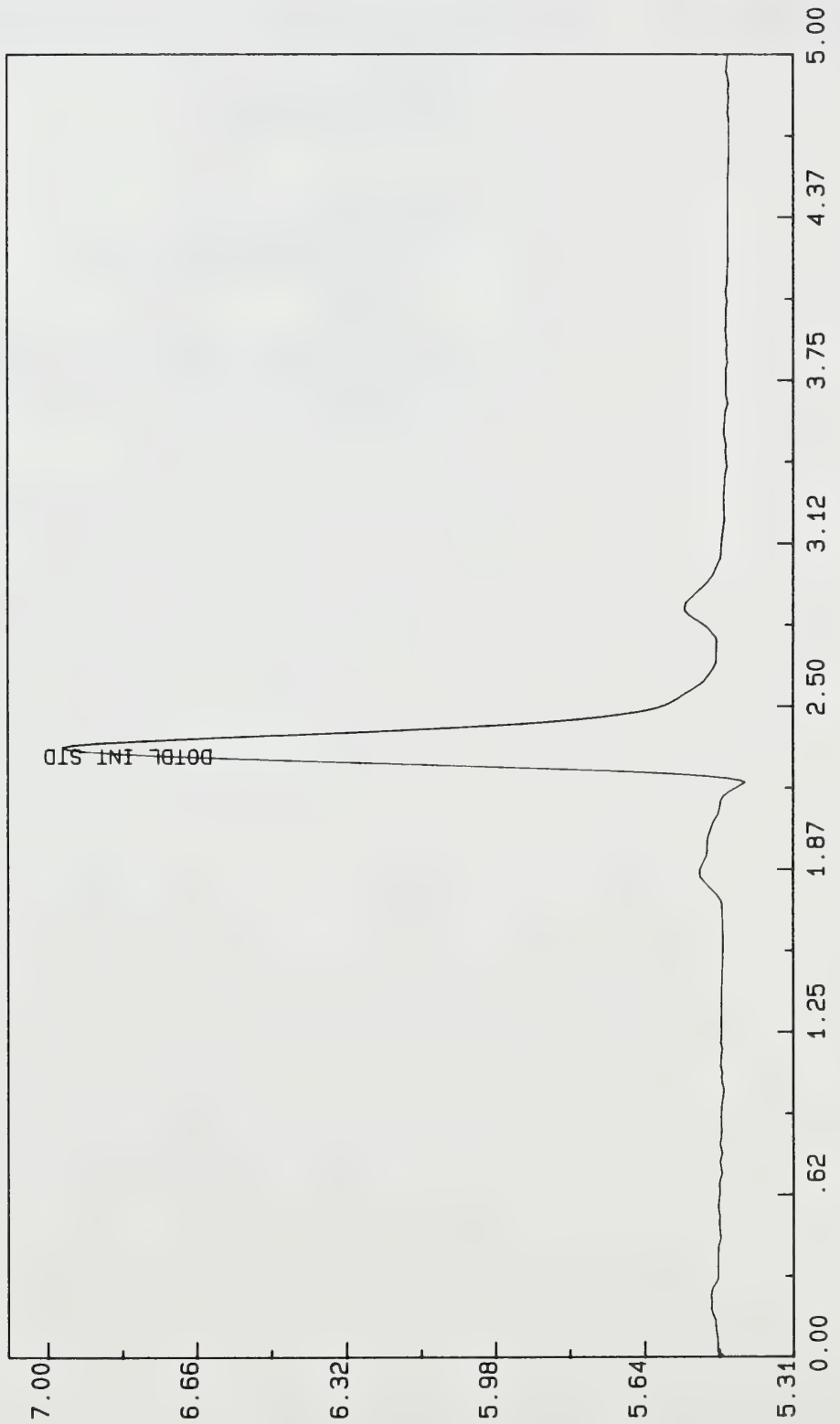
Instrumental Settings and Conditions

The following parameters may be modified as necessary to compensate for daily variations in instrument performance characteristics.

Mobile phase	Hexane:ethyl acetate (95:5) containing 3% acetic acid
Column	See Section B.2
Column flow rate	2.5 mL/min
Post column derivatization reagent	0.005% morin in ethanol
Derivatization reagent flow rate	1.3 mL/min
Fluorescence detector settings	Excitation at 420 nm Emission at 495 nm
Retention time	Approximately at 3.0 min

DETERMINATIVE METHOD

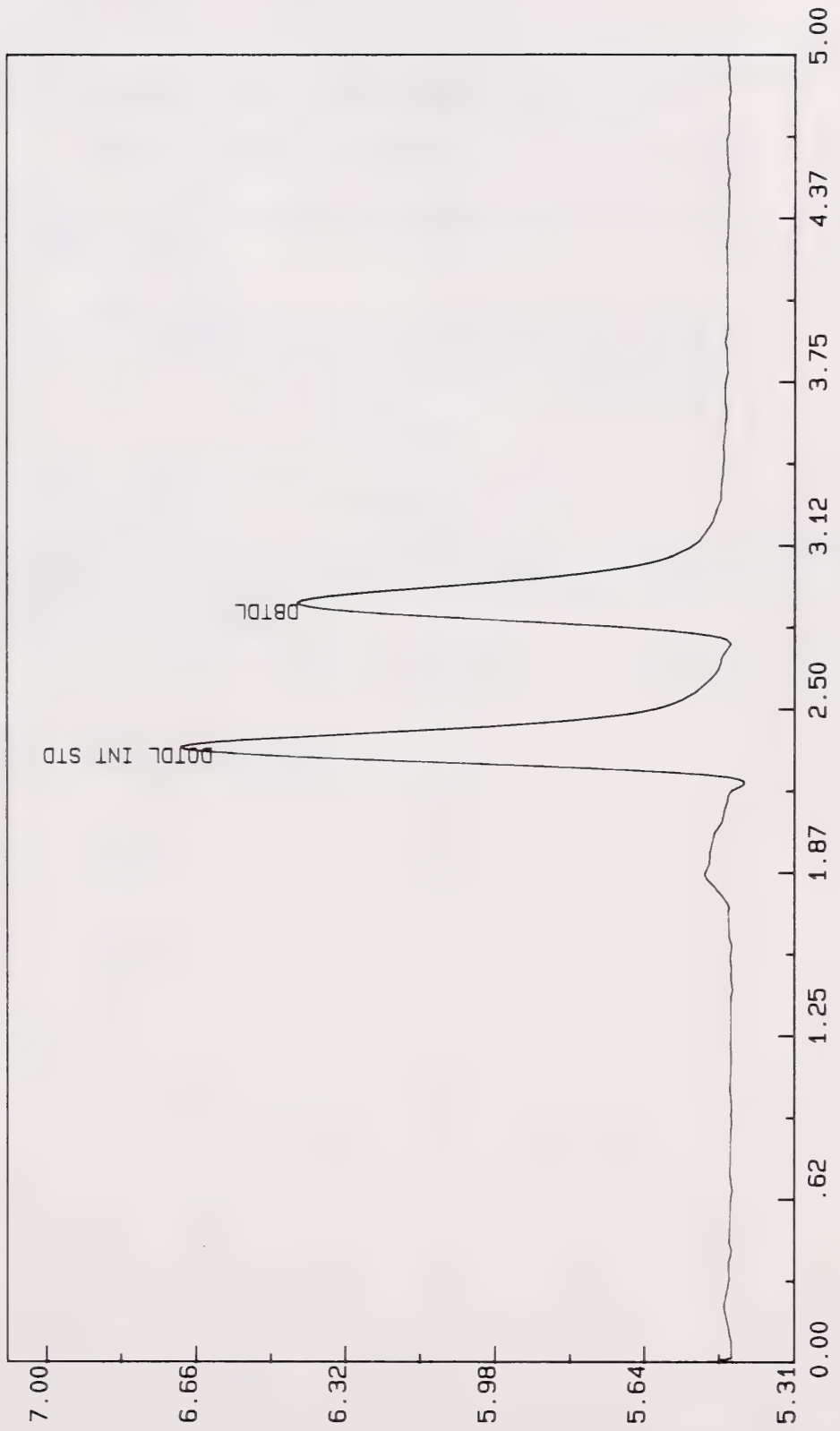
F. ANALYTICAL QUANTITATION (Continued)



DBTDL—Tissue Blank

DETERMINATIVE METHOD

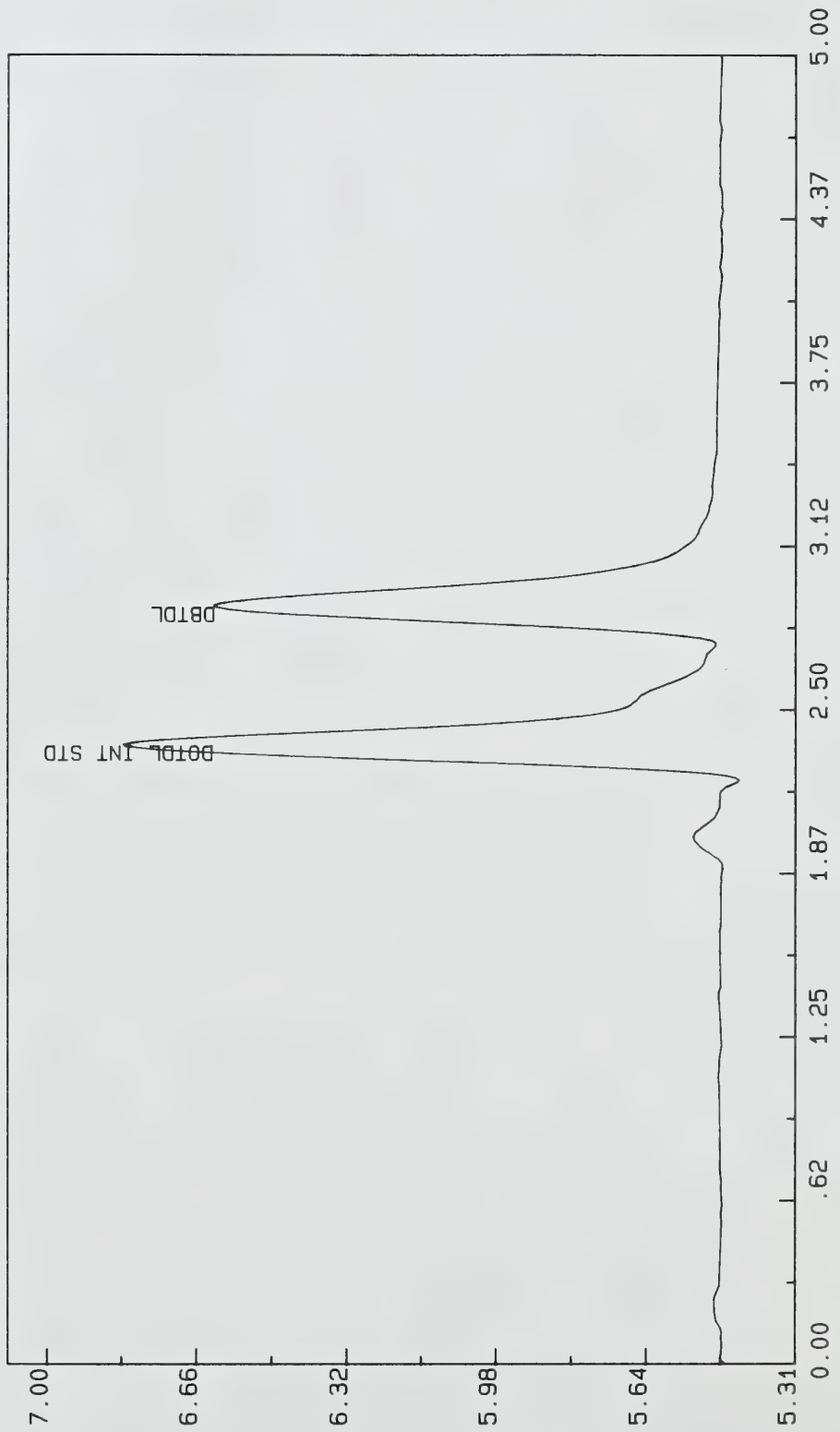
F. ANALYTICAL QUANTITATION (Continued)



DBTDL Rec. @ 0.5 ppm

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



DBTDL Ext. Std. @ 0.5 ppm

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Construct a standard curve from the three fortified samples and tissue blank as follows:

a. Measure peak heights of DBTDL and the internal standard in each fortified sample and calculate their respective peak height ratios.

DBTDL peak height/DOTDL (I.S.) peak height

b. Using linear regression, construct a standard curve of DBTDL concentration vs. peak height ratio.

The equation is $y = mx + b$
where x = DBTDL concentration (ppm)
y = DBTDL/I.S. peak height ratio
m = slope
b = y intercept

The correlation coefficient (r) should be $\geq .995$.

c. Using the regression slope and intercept, compute the DBTDL concentration (x) for each incurred sample from the peak height ratio.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Quantitation of Dibutyltin in Poultry Tissues

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

		Hazards	*Recommended Safe Procedures*
C. Reagents			
	Hexane Ethyl acetate Ethanol	Flammable. May be irritating to respiratory tract.	All work should be performed in an efficient hood and away from heat-generating devices.
	Hydrochloric acid Acetic acid	Will produce burn and irritations to the skin, eyes, and respiratory system.	Same as above.

4. Disposal Procedures

Organic solvents	See above.	Dispose of in proper container and segregate chlorinated from nonchlorinated waste drums.
Acid solution	See above.	Flush down sink followed by lots of water.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Dibutyltin Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

DIBUTYLTIN ANALYSIS

Regression Analysis for y = mx + b
r =
s =
m =
b =

Analyst: _____ Date: _____

HPLC Analytical Operating Conditions:

Adsorbosphere CN 15 cm X 4.6 mm 5 μ column

Mobile phase—hexane:ethyl acetate (95:5) (V/V) containing 3% acetic acid
Flow—2.5 mL/min

Post column derivatization reagent—0.005% morin in ethanol
Flow—1.3 mL/min

Injection volume—80 μL

Sample Identification

Critical Control Points

Procedure Key	Sample Weight (grams)	Spiking Volume (μL)	Shake Time (min)	Rotary Evap. Temp. (° C)	Rotary Evap. Vol. (mL)	N-Evap Temp. (° C)	Peak Height (mm)	ppm or % Rec. Found	Comments
STD 0.25 ppm									
STD 0.50 ppm									
STD 1.00 ppm									
Recovery @ 0.5 ppm									
Control Blank									

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Analyte	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*
DBTBL	0.25-5.0	72-110	≤ 15 at 0.25 ppm

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5.0 ± 0.1 g
b. Spiking volume	100 ± 1μL
c. Shake time (Shaker)	10 ± 2 minutes
d. Rotary Evap. temperature	35 ± 2°C
e. Rotary Evap. volume	1.0 ± 0.5 mL (do not go to dryness)
f. N-Evap temperature	35 ± 2° C

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Duplicate sets of standard curve on each of three consecutive days at 0, 0.25, 0.50, and 1.00 ppm.

ii. Phase II: Self-fortified samples—Using turkey liver tissue, spiked at 0, 0.25, 0.50, and 1.00 ppm. Analyses are performed in duplicate on three consecutive days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents:

i. Frequency: At least one check sample biweekly per analyst.

ii. Random replicates or blind check samples may be chosen by the supervisor or Laboratory QA Officer.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Turkey liver and muscle.

b. Sample receipt size: Approximately 50 g.

c. Condition upon receipt: Chilled to frozen (< 10° C).

d. Sample storage:

i. Time: 3 months.

ii. Condition: ≤ 4° C.

6. Sample Set

Each set must contain a tissue blank, a tissue blank fortified with 0.5 ppm of DBTDL, and up to 6-10 additional samples per set.

7. Sensitivity

a. Lowest detectable level (LDL): 0.10 ppm.

b. Lowest reliable quantitation (LRQ): 0.10 ppm.

c. Minimum proficiency level (MPL): not determined.

ANALYSIS OF DECOQUINATE RESIDUES IN ANIMAL TISSUES USING ZYMATE ROBOTIC SYSTEM

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 10
G. Calculations 14
H. Hazard Analysis 15
I. [Reserved]
J. Quality Assurance Plan 16

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Decoquinate is used in various animal species for the control of bacterial infections. It is primarily used in cattle, swine, and poultry to control intestinal infections.

Residue is extracted from tissue homogenate using a methanol/chloroform extracting solvent system. The homogenate is centrifuged to remove solid material, and an aliquot of the solvent phase is taken. This aliquot is extracted with 5% aqueous metaphosphoric acid to remove interfering materials. The extract is again centrifuged to separate the liquid phases and an aliquot of the lower layer (chloroform) is injected onto a C_{18} reversed phase liquid chromatographic column. Decoquinate is detected by measuring fluorescence at 390 nm. Calcium ion is present in the mobile phase to promote fluorescence of the decoquinate, which is not naturally fluorescent.

2. Applicability

This method is applicable to liver and muscle tissues of bovine, porcine, and avian species.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. 50 mL polypropylene centrifuge tubes.

b. 16 × 100 mm culture tubes, Scientific Products Cat. No. T1290-6, or equivalent.

c. 5 mL pipet tips, Oxford type #814, or equivalent.

d. 1 mL pipet tips, Elkay #0000-025, or equivalent.

e. Depth filters for 1 mL pipet tips, Zymark #39247.

2. Instrumentation

a. LC System consisting of the following:

i. Waters 510 solvent pumping system.

ii. Waters radial compression module (Waters Asso. RCM-100).

iii. C_{18} 10 μm radial compression column (Waters Asso.).

iv. Waters 420-AC Fluorescent Detector.

(a) 400 nm emission filter.

(b) 360 nm excitation filter.

v. Spectra Physics model SP-4270 computing integrator.

b. Zymark Robotics System (Pytechnology System).

i. ZP-100 Zymate II Core System.

ii. Z-710 centrifuge section.

iii. Z-310 LC inject section.

iv. ZP-620-6 dilute and dissolve section (includes vortex mixer).

v. ZP-013-7 50 mL centrifuge tube rack sections (2 each).

vi. ZP-013-3 16 × 100 mm test tube rack section.

vii. ZP-470-3 liquid/liquid extraction section.

vii. Tissuemiser section (custom manufactured by Zymark, Inc.).

viii. ZP-911-1 pipeting hand (1-4 mL).

ix. ZP-910-1 pipeting hand (0.2-1.0 mL, with filters attached).

x. ZP-900-2 gripper hand for 50 mL centrifuge tubes.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

xi. ZP-900-1 gripper hand for 16 × 100 mm test tubes.

xii. Disposal section.

xiii. ZP-510 Master Lab stations (2 each).

xiv. ZP-830 power and event controller.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Chloroform, HPLC grade, MCB OmniSolv, or equivalent.

b. Methanol, HPLC grade, Baker ResiAnalyzed, or equivalent.

c. Metaphosphoric acid, Aldrich Chemical #23,927.5, or equivalent.

d. Calcium nitrate, $Ca(NO_3)_2{:}4H_2O$, Baker reagent grade, or equivalent.

2. Solution List

a. Extraction solvent, methanol/chloroform, 4:1 (v/v).

b. 5% aqueous solution of metaphosphoric acid.

c. HPLC mobile phase, 23.6 g $Ca(NO_3)_2{:}4H_2O$, 100 mL distilled deionized water, 50 mL acetonitrile, 850 mL methanol. Filter prior to use.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Decoquinate analytical reference standard > 99% purity can be obtained from:
Hess and Clark
Division of Richardson-Merrell, Inc.
Ashland, OH 44805

2. Preparation of Standards

a. Stock standard solution (50 μg/mL).

Weigh 5.0 mg $\pm$ 0.1 mg decoquinate and dissolve in 100 mL methanol.

b. HPLC reference standards.

i. Pipet 1.0 mL of stock standard solution into a clean 100 mL volumetric flask. Dilute to volume with methanol. Concentration is 0.5 μg/mL.

ii. Pipet 2.0 mL of stock standard solution into a clean 100 mL volumetric flask. Dilute to volume with methanol. Concentration is 1.0 μg/mL.

iii. Pipet 4.0 mL of stock standard solution into a clean 100 mL volumetric flask. Dilute to volume with methanol. Concentration is 2.0 μg/mL.

3. Storage Conditions

a. Store stock reference solution at 4° C.

b. HPLC reference solutions may be stored at room temperature.

4. Shelf Life Stability

a. Stock standard solutions are stable for 6 months at 4° C.

b. Reference solutions must be freshly made every month.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Allow frozen tissue to thaw.

2. Sample Extraction

a. Weigh 5.0 ± 0.1 g of tissue into a 50 mL disposable centrifuge tube.

b. Add 20 mL extraction solvent (4:1 methanol/chloroform).

c. Centrifuge for 5 min at 2000 rpm.

d. Transfer 12 mL (3.0 gram tissue equivalent) to a clean 50 mL centrifuge tube.

e. Add 10 mL metaphosphoric acid and 3.0 mL chloroform; vortex for 90 seconds.

f. Centrifuge for 5 minutes at 2000 rpm to completely separate organic layer.

g. Transfer 3.0 mL of the lower organic layer to a clean 16 X 100 mm glass test tube.

h. Pipet 1.0 mL of this extract through a disposable depth filter.

i. Inject 20 μL of the filtered extract for HPLC analysis. Inject 20 μL of HPLC standards for construction of standard curve.

The above analysis is completely described by the program DECO, which was written for the Zymate Pytechnology robotic system. This program is shown in E.3.

3. DECO

EASYLAB PROGRAM LISTING

```
    ERASE
    PROMPT
    PROMPT How many samples in this set?
    INPUT TOTAL.SAMPLES {GET # OF SAMPLES FROM OPERATOR}
    SAMPLE.NUMBER = 1
    SET INITIAL CONDITIONS
10  RACK.7.INDEX = SAMPLE.NUMBER
    RACK.8.INDEX = SAMPLE.NUMBER
    RACK.2.INDEX = SAMPLE.NUMBER
    GET.A.SAMPLE {PICK UP SAMPLE FROM RACK 7}
    MLS.2.VOLUME.A = 20 {EXTRACTION SOLVENT VOLUME}
    MLS.2.TIME.A = 40
    VORTEX.TIME = 0
```

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

```
    DILUTE.AND.DISSOLVE.2 {INSTITUTE SOLVENT DELIVERY}
    HOMOGENIZE.SAMPLE {BLEND SAMPLE}
20  CENTRIFUGE.INDEX = 1
    PUT.TUBE.IN.CENTRIFUGE {LOAD CENTRIFUGE}
    TIMER (6) = 300 {SET 5 MIN CENTRIFUGE TIME}
    WASH.TISSUMISER {WASH TISSUMISER}
    WAIT FOR TIMER (6) {WAIT FOR REMAINING CENTRIFUGE TIME}
30  GET.TUBE.FROM.CENTRIFUGE {UNLOAD CENTRIFUGE}
    PUT.TUBE.IN.RACK.7 {REPLACE TUBE IN RACK.7}
    GET.5ML.TIP {ATTACH A 5 ML PIPET TIP ON SYRINGE HAND}
    DO 3 TIMES
    MOVE.OVER.RACK.7
    PIPET.VOLUME = 4.0 {TRANSFER 4 ML EXTRACT TO CLEAN
         TUBE}
    ASPIRATE.5ML.TIP
    MOVE.OVER.RACK.8
    C.CAPPED = 0
    DISPENSE.5ML.TIP
    ENDDO {REPEAT TRANSFER FOR A TOTAL OF 12 ML}
    DISPOSE.TO WASTE {DISCARD PIPET TIP}
35  GET.TUBE.FROM.RACK.8 {PICK UP SECOND TUBE}
    S:C.CAPPED = 0
    DISPENSE.CHCL3 {ADD 3.0 ML CHLOROFORM TO EXTRACT}
    MLS.2.VOLUME.B = 10 {SET VOLUME OF METAPHOSPHORIC
         ACID}
    VORTEX.TIME = 60 {SET VORTEX TIME TO 60 SECONDS}
    DILUTE.AND.DISSOLVE {INITIATE ACID DELIVERY AND
         VORTEXING}
    PUT.TUBE.IN.CENTRIFUGE {LOAD SAMPLE INTO CENTRIFUGE}
    TIMER = 6 {SET 5 MIN TIMER FOR CENTRIFUGE STEP}
    WAIT FOR TIMER (6)
30  GET.TUBE.FROM.CENTRIFUGE {UNLOAD CENTRIFUGE}
    LL.CANNULA.DEPTH = 11.5 {SET DEPTH FOR LIQUID REMOVAL}
    LL.CANNULA.VOLUME = 3.0 {SET VOLUME TO REMOVE}
    ASPIRATE.LL.CANNULA {INITIATE REMOVAL}
```

DETERMINATIVE METHOD

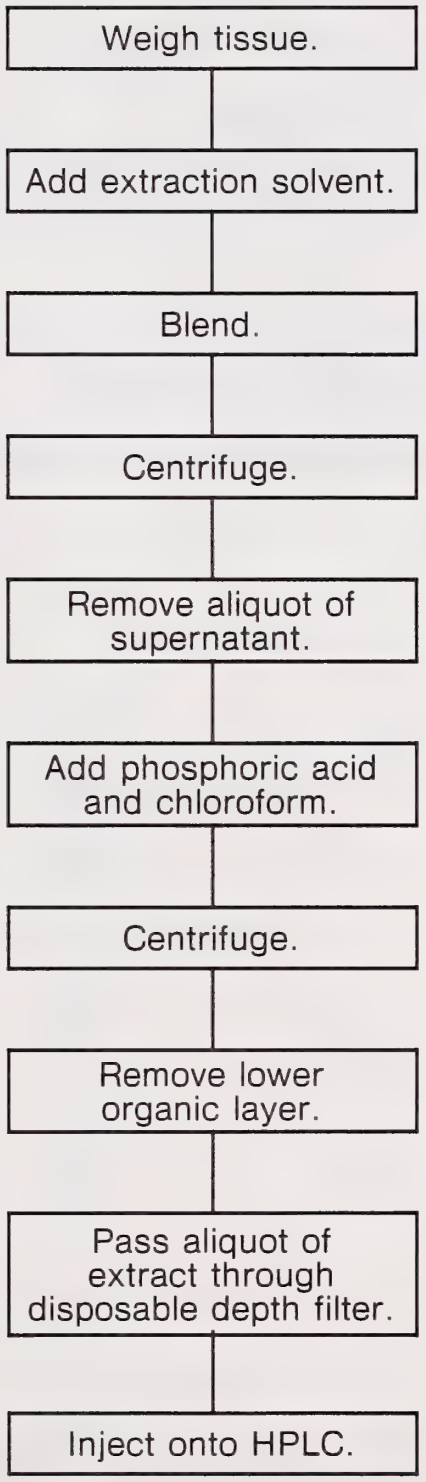
E. EXTRACTION PROCEDURE (Continued)

```
PUT.TUBE.IN.RACK.8 {RETURN TUBE TO HOLDING RACK}
GET.TUBE.FROM.RACK.2 {GET CLEAN 16 × 100 mm TUBE}
DISPENST.LL.CANNULA {DISPENSE 3.0 ML EXTRACT}
PUT.TUBE.IN.RACK.2 {RETURN TUBE AND EXTRACT TO RACK.2}
GET.1ML.TIP {ATTACH 1 ML PIPET TIP ON SYRINGE HAND}
MOVE.OVER.RACK.2 {PREPARE TO REMOVE 1.0 ML FOR LC}
PIPET.VOLUME = 1.0 {SET VOLUME TO REMOVE}
ASPIRATE.1ML.TIP {INITIATE PIPET STEP—ALSO DISCARDS
    FILTER}
SEP.TIME = 8 {SET LC RUN TIME TO 8 MIN}
LC.INJECT {INITIATE LC INJECTION}
DISPOSE.TO.WASTE {DISCARD USED TIP}
WASH.LL.CANNULA {RINSE L/L CANNULA}
SAMPLE.NUMBER = SAMPLE.NUMBER + 1
IF SAMPLE.NUMBER < = TOTAL SAMPLES THEN GOTO 10
PARK.HAND$ {FINISHED—PARK HAND AND QUIT}
```

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

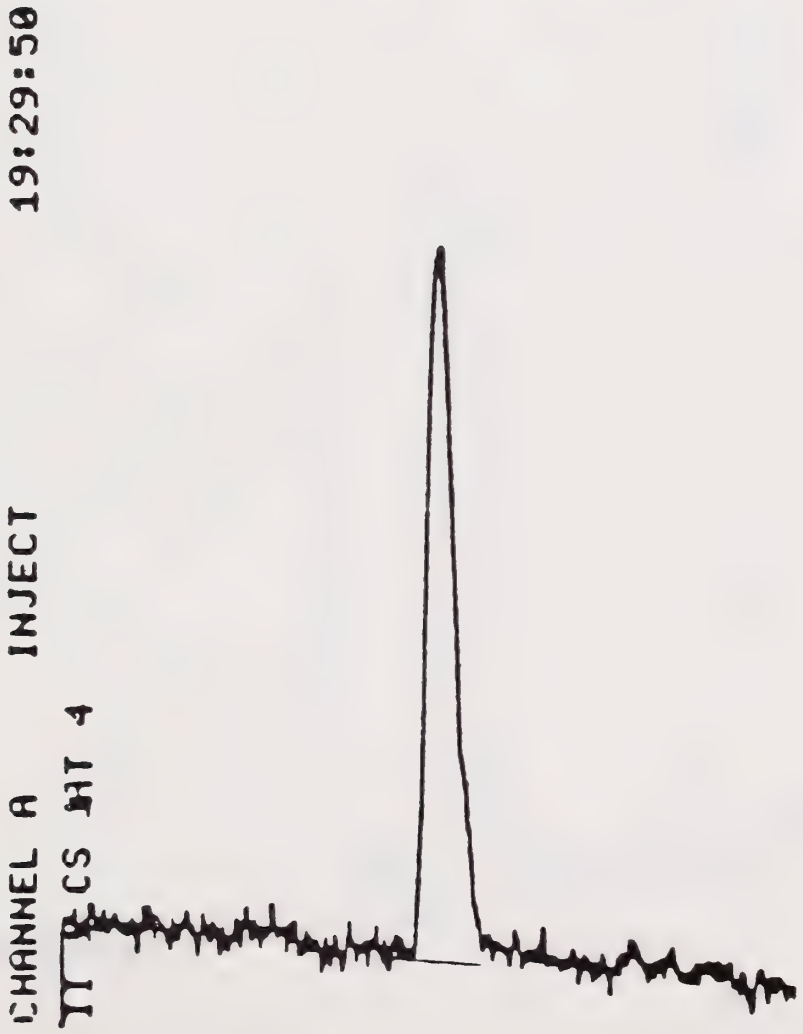
1. Instrumental Settings and Conditions	a. Detector gain:	32
	b. Integrator attenuation:	4
	c. Chart speed:	1.0 cm/min
	d. Fluorescent detector:	360 nm excitation filter 400 nm emission filter
	e. Mobile phase flow rate:	2.0 mL/min
2. Interferences	No known interferences.	
3. Sample Chromatograms	Chromatograms for standard, blank liver, and fortified liver are presented.	

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

DECOQUINATE STANDARD
20 μL Injected
Concentration = 1.0 ng/μL

2.0 mL/min flow rate
0.05M $Ca(NO_3)_2$ methanol/water/acetonitrile (85/10/5)

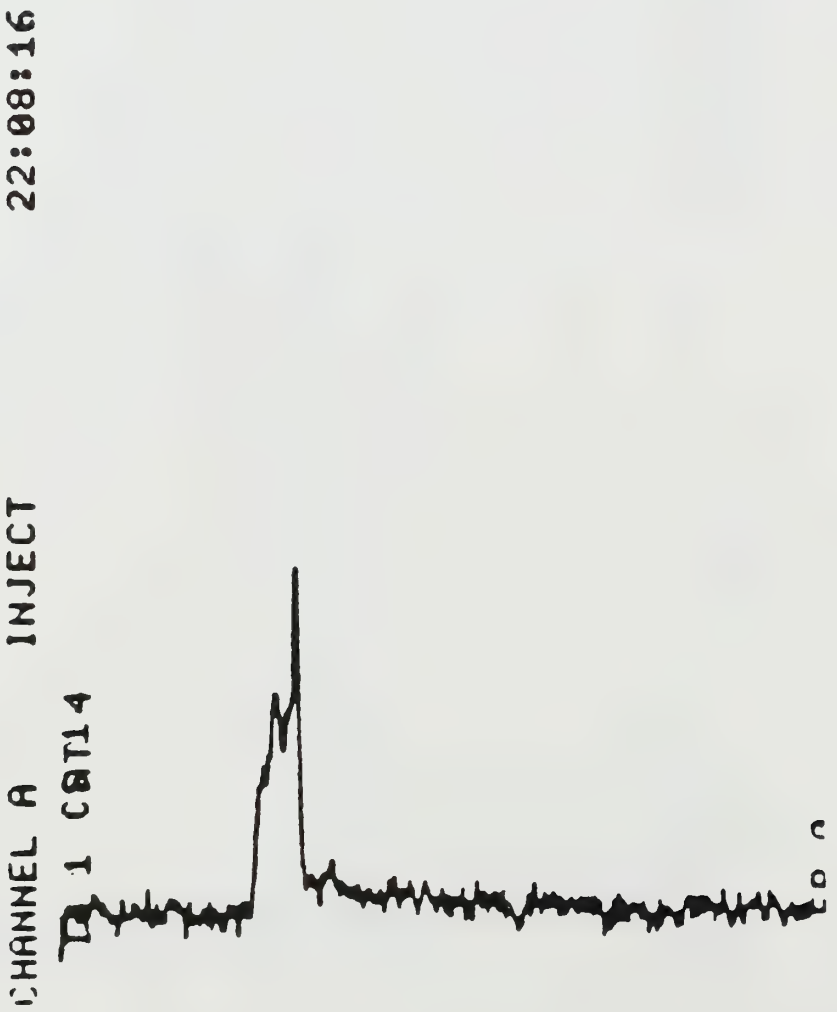


DETERMINATIVE METHOD

F. ANALTYICAL QUANTITATION (Continued)

DECOQUINATE LIVER BLANK
20 μL Injected
Concentration = 1.0 ng/μL

2.0 mL/min flow rate
0.05M $Ca(NO_3)_2$ methanol/water/acetonitrile (85/10/5)

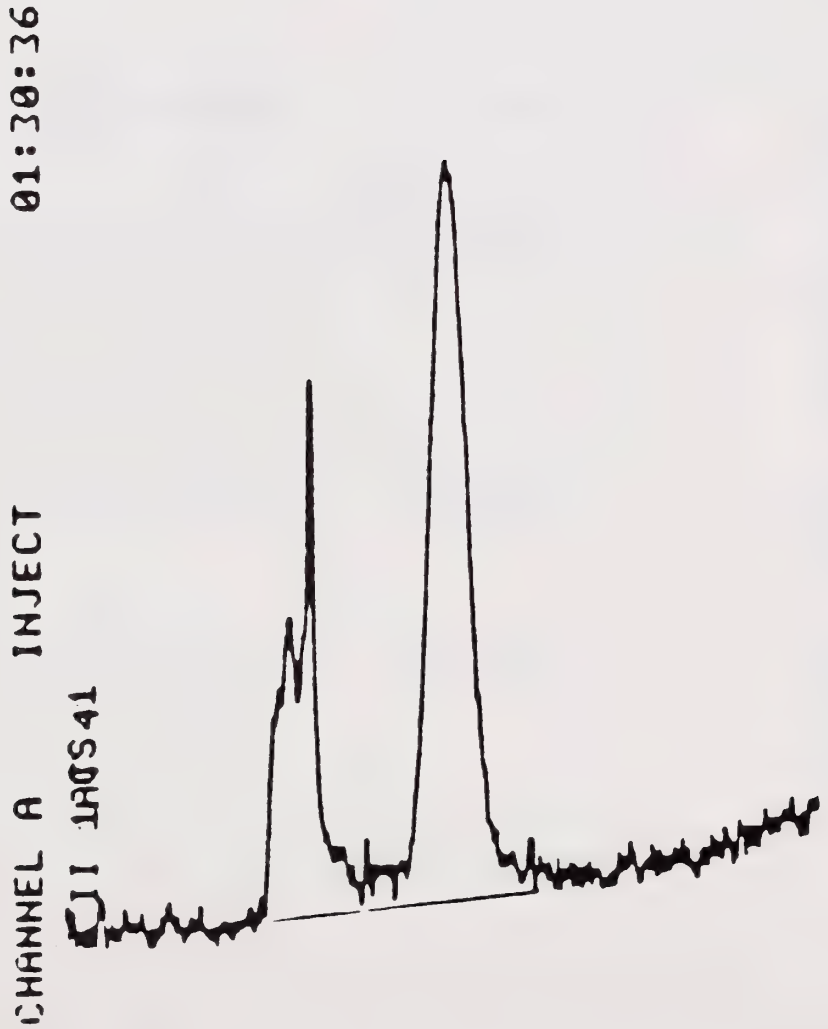


DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

DECOQUINATE LIVER SPIKED WITH 2.0 PPM
20 μL Injected
Concentration = 1.0 ng/μL

2.0 mL/min flow rate
0.05M $Ca(NO_3)_2$ methanol/water/acetonitrile (85/10/5)



DETERMINATIVE METHOD

G. CALCULATIONS

Procedure	Measure the peak height of the HPLC standards and calculate the least squares linear regression calibration line using the methodology described in Section 1.5.67 (July 9, 1982) of the Chemistry Quality Assurance Handbook. Measure the peak height of each unknown sample and calculate the amount of decoquinate using the calibration line.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Analysis of Decoquinate Residues in Animal Tissues Using Zymate Robotic System

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Chloroform Methanol	Flammable and may produce toxic effects to the skin, eyes, and respiratory system. Vapors are hazardous.	Work in efficient fume hood, away from heaters. Use plastic gloves.
Metaphosphoric acid	Corrosive.	Wear eye protection, lab coat, and protective gloves.

4. Disposal Procedures

Organic solvents	See above	Store in organic solvent disposal container until disposed of by a contractor or in-house specialist.
Acidic solutions	See above	Dilute and flush into an acid disposal sink located in a well-ventilated area.
Chloroform	See above	Store with waste chlorinated solvents until disposed of by a contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Analyte	*Analytical Range (ppm)**	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
Decoquinate	0-4.0	80-110	10	15*

* Estimated value; actual value to be determined after validation survey.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight	5.0 ± 0.1 g
Volume of metaphosphoric acid	9.0 ± 0.2 mL
Volume of extract	12.0 ± 0.2 mL
Volume of extraction solvent	20.0 ± 0.2 mL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Duplicate sets of standard curves on each of three days at 0, 0.5, 1.0, 2.0, and 4.0 ppm.

ii. Phase II: Self-fortified recovery samples using beef liver tissue spiked at 0, 0.5, 1.0, 2.0, and 4.0 ppm (duplicates) on three days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation. Twelve samples submitted by FSIS Science & Technology Western Laboratory (or laboratory LSO if only one laboratory is performing this test).

b. Acceptabilty criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst. Blind samples or random duplicates chosen by the supervisor or LSO.

ii. Records to be maintained by analyst and reviewed by supervisor and LSO:

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

(d) Running average, standard deviation, and CV for all recoveries.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability Criteria

i. CV equal to or better than that stated in J.1.

ii. CV for running average of the percent recoveries less than 10%, with all recoveries to range as stated in J.1.

iii. If unacceptable values are obtained, then:

(a) Stop all official analyses for that analyst.

(b) Investigate and identify probable cause.

(c) Take corrective action.

(d) Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix of choice: Bovine, porcine, and avian liver and muscle.

b. Sample receipt size, minimum: Sufficient for all quantitative and confirmation analyses and sample reserve (500 g).

c. Condition upon receipt: Chilled or frozen.

d. Sample storage.

i. Time: 3 months.

ii. Condition: Frozen at −4° C or lower until analyzed.

6. Sample Set

a. Blank.

b. Recovery.

c. Standards.

d. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 0.2 ppm.

b. Lowest reliable quantitation (LRQ): 0.5 ppm.

c. Minimum proficiency level (MPL): 0.5 ppm.

DEXAMETHASONE

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 10
G. Calculations 13
H. Hazard Analysis 14
I. Worksheet 15
J. Quality Assurance Plan 19

II. Confirmatory Method

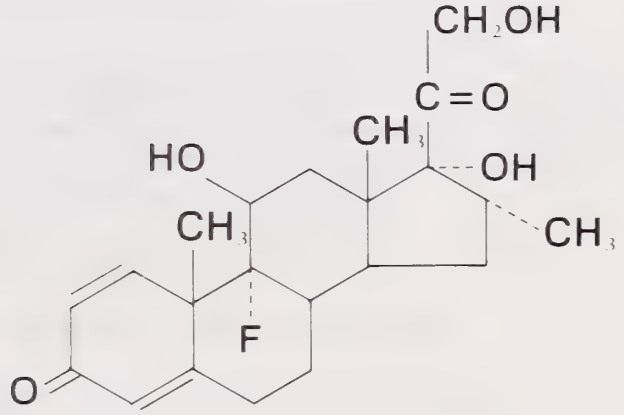
A. Introduction 22
B. Equipment 23
C. Reagents and Solutions 24
D. Standards 24
E. Derivatization 25
F. Analytical Confirmation Procedure 27
G. [Reserved]
H. Hazard Analysis 29
I. Worksheet 30
J. Quality Assurance Plan 33

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Liver and muscle tissue are extracted with a mixture of aqueous buffer and acetonitrile. Addition of hexane and dichloromethane to this extract produces a three-phase liquid system that provides a crude separation of compounds into three polarity classes. The layer containing dexamethasone is collected and subjected to coupled-column normal-phase HPLC, which performs further sample clean-up on-line with analytical determination. Time-programmable switching valves control the chromatographic events. The sample is injected onto the first column. For the time dexamethasone is known to elute, the flow from column 1 is diverted to a second column that collects dexamethasone along with any co-eluting matrix components. The contents of column 2 are then backflushed with a stronger eluent onto a third column for the final separation, which isolates dexamethasone. Meanwhile, column 1 is regenerated, i.e., retained polar materials are removed with a more polar eluent. [T. M. P. Chichila, P. O. Edlund, J. D. Henion, *Journal of Chromatography*, Biomedical Applications, 488 (1989)].



Dexamethasone
MW = 392.20

2. Applicability

This method will determine dexamethasone at concentrations above 10 ppb in bovine liver and muscle.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

If necessary, an equivalent may be substituted for all apparatus listed below.

a. Homogenizer—Waring Blender—VWR Scientific.

b. 50 mL screw-cap polypropylene centrifuge tubes—Corning.

c. Pasteur disposable pipets, 10 mL.

d. Vortex mixer—Fisher Scientific Touch-Mixer.

e. Eppendorf pipets.

f. Disposable pipets.

g. pH meter—Fisher Scientifc Model 815MP.

h. Roto-Rack—Fisher Scientific.

i. Constant temperature water bath (37° C)—American Optical Corp.

j. Centrifuges

   i. Damon IEC Model K, equipped with 12-place angle rotor and capable of achieving a g force up to 4500.

   ii. Fisher Micro-Centrifuge Model 59A.

k. Glass screw-cap test tubes, 25 mm i.d. × 150 mm.

l. Scintillation Vials, 20 mL—Wheaton.

m. Reacti-Therm Dry Block Heater with custom-drilled blocks—Pierce Chemical Co.

n. Volumetric flask, 10 mL.

o. Centrifugal filters—Microfilterfuge Tubes, 0.45 μm nylon—Rainin Instrument Co.

p. Graduated cylinders, 100 mL and 1 L.

q. Autosampler vials—1 mL size with conical bottom.

r. Ultrasonic bath—Metler Electronics Corp. Cavitator.

s. Disposable centrifuge tubes, 5 mL, screw-cap, with conical bottom—VWR Scientific.

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

2. Instrumentation

a. Perkin Elmer Model ISS-100 autosampler with 50 μL sample loop.

b. Three Waters HPLC pumps. Model 500 and 600A.

c. Two Waters air-actuated automatic switching valves.

d. A Waters temperature control module to maintain columns 1 and 3 at 30° C.

e. A Hewlett-Packard Model 3390A integrator.

f. A Waters Model 680 gradient control module to program the pumps, switching valves, and integrator.

g. Columns:

i. Column 1: Spherisorb phenyl of lot #23/163, 4.6 mm i.d. × 50 mm, 3μm, packed by Keystone Scientific (State College, PA).

ii. Column 2: Silica, 4 mm i.d. × 12.5 mm, 5 μm, packed by MacMod Analytical (Chadds Ford, PA).

iii. Column 3: Spherisorb nitrile, 4.6 mm i.d. × 100 mm, 3 μm, packed in-house. With slight adjustment of mobile phase strength, the commercially packed equivalent of this column (same lot not necessary) will provide the same operation.

I. DETERMINATIVE METHOD

C. REAGENTS

Reagent List

a. B-Glucuronidase, Type H-2S—Sigma Chemical Corp. Activity: 133,000 units/mL.

b. Acetic acid, reagent grade—Fisher Scientific.

c. Acetonitrile, dichloromethane, ethyl acetate, hexane, methanol, 2-propanol, sodium acetate, water: HPLC grade—Fisher Scientific.

d. 0.04 M sodium acetate buffer: Weigh 5.44 g sodium acetate and dissolve in 1 L of HPLC-grade water.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Dexamethasone analytical standard—Sigma Chemical Corporation, St. Louis, MO.

2. Preparation of Standards

a. Stock standard, 100 μg/mL: Dissolve 10.0 mg of dexamethasone stock standard in 100 mL methanol.

b. Intermediate standard, 10 μg/mL (or 10 ng/μL): Dilute 10 mL of the stock solution in 100 mL volumetric flask, using methanol.

c. Fortification standard: Dilute the following standards in ethyl acetate:

mL of Intermediate Standard	*Final Volume (mL)*	*Solution Concentration*
1	10	1 ng/μL
2	10	2 ng/μL
3	10	4 ng/μL

d. HPLC standard, 0.2 ng/μL: Dilute 0.2 mL of intermediate standard solution to 10 mL using HPLC mobile phase solution.

3. Storage Conditions

All samples must be stored in the freezer at −10° C until ready to use.

4. Shelf Life Stability

Not known at the present time. Peak signal of the same standard injected at different time has to agree within ± 10%. If not, prepare new standard.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

a. Weigh 5.0 g homogenized beef liver or beef muscle into 50 mL polypropylene centrifuge tube.

   NOTE: Tissue should be kept frozen (– 10° C) at all times until assayed.

b. With 10 mL disposable pipet, add 10 mL of 0.04 M sodium acetate buffer.

c. Vortex mix for 30 seconds.

d. Using Eppendorf pipet, adjust pH to 4.2-4.7 with 75 μL glacial acetic acid, swirl, and check with pH meter.

e. For liver or muscle fortification curve for linear regression analysis, add 100 μL of the following solutions of dexamethasone in ethyl acetate to achieve the desired tissue concentration of dexamethasone. For example, in liver:

Tissue Concentration	*Solution Concentration*
0 ppb	0 ng/μL
20 ppb	1 ng/μL
40 ppb	2 ng/μL
80 ppb	4 ng/μL

   NOTE: Separate curves are constructed using liver and muscle and only the same kind of tissue samples are analyzed by each curve.

f. Add 100 μL of B-glucuronidase to each sample. Cap and mix by rotation (7 rotations/minute for 3 minutes on Roto-Rack).

g. Incubate for 8 hours at 37° C with gentle shaking in water bath.

h. After incubation, add 20 mL of acetonitrile to each tube and mix by rotation. For liver, 7 rotations/minute for 10 minutes. For muscle, 35 rotations/minute for 30 minutes and vortex-mix for 30 seconds.

i. Centrifuge samples: liver for 10 minutes at a g force of 3600, muscle for 30 minutes at a g force of 4500.

   NOTE: g force = (1.118×10^{-5})(radius of rotation, cm)$(RPM)^2$

   radius of rotation = distance from center of shaft to furthest inside tip of the tube.

j. Decant supernatant into glass test tubes (25 mm i.d. × 150 mm).

   NOTE: Liquid should pour off quite cleanly from plug, but if unavoidable, a small amount of decanted debris will not interfere.

   Stop Point: Samples may be stored at 4° C for as long as 12 hours, perhaps longer. Allow them to return to room temperature before proceeding.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

k. With 10 mL disposable pipets, add 8 mL of hexane and 2 mL of dichloromethane to the supernatant.

l. Roto-Rack (7 rotations/minute) for 3 minutes.

m. Centrifuge at a g force of 1200 for 4 minutes.

n. With 10 mL disposable pipets, transfer 15 mL of the middle layer to a scintillation vial, leaving 1-2 mL of the middle layer behind to avoid contamination by the other layers.

o. Evaporate samples to dryness under nitrogen at 60° C.

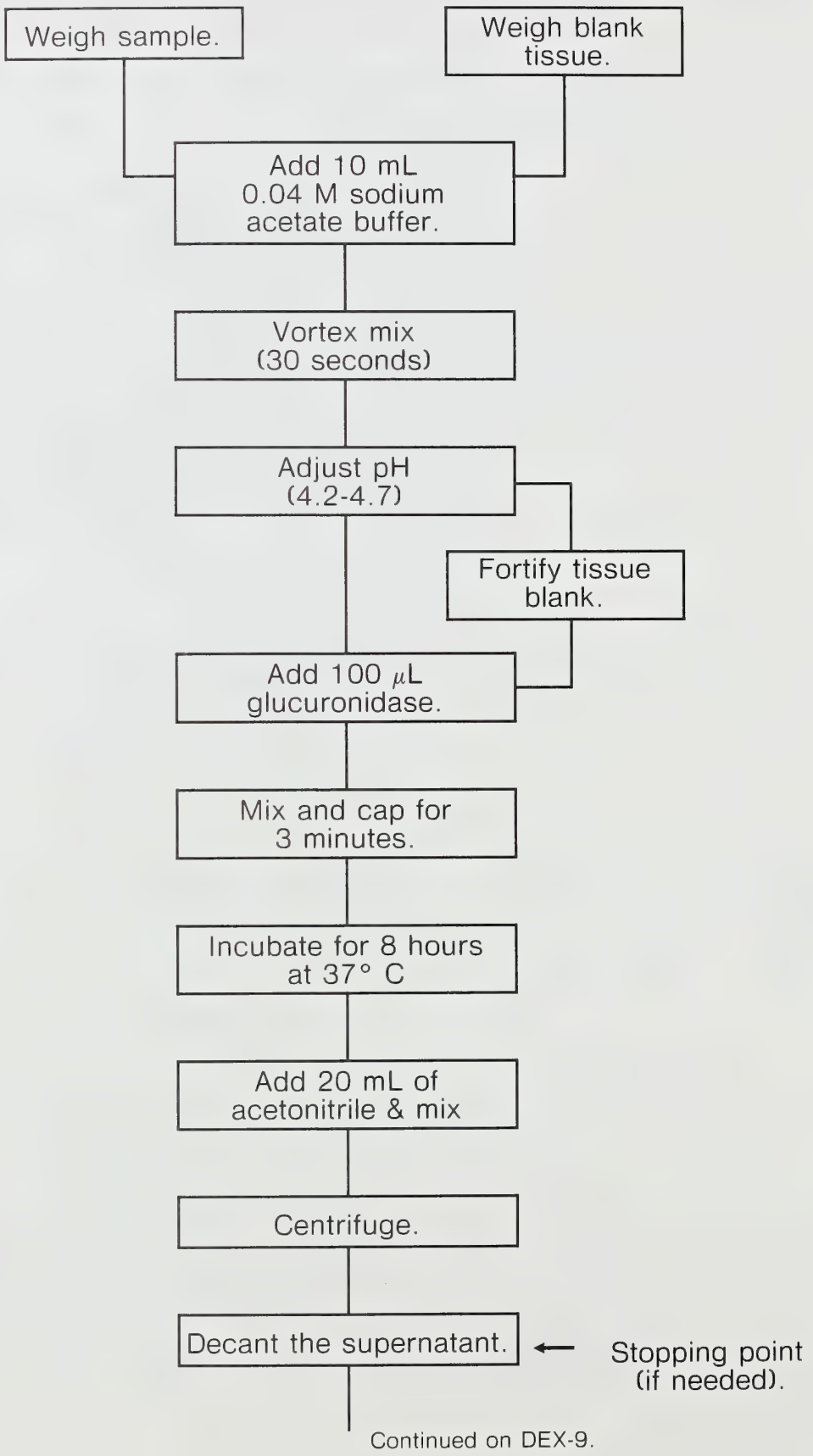
NOTE: All acetonitrile must be removed; this takes 45-60 minutes.

Stop Point: If time permits, proceed to section F. Otherwise, resulting residues can be stored in the freezer. Stability of dexamethasone and matrix components in these residues was not studied, but no degradation was observed after several days of freezer storage.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

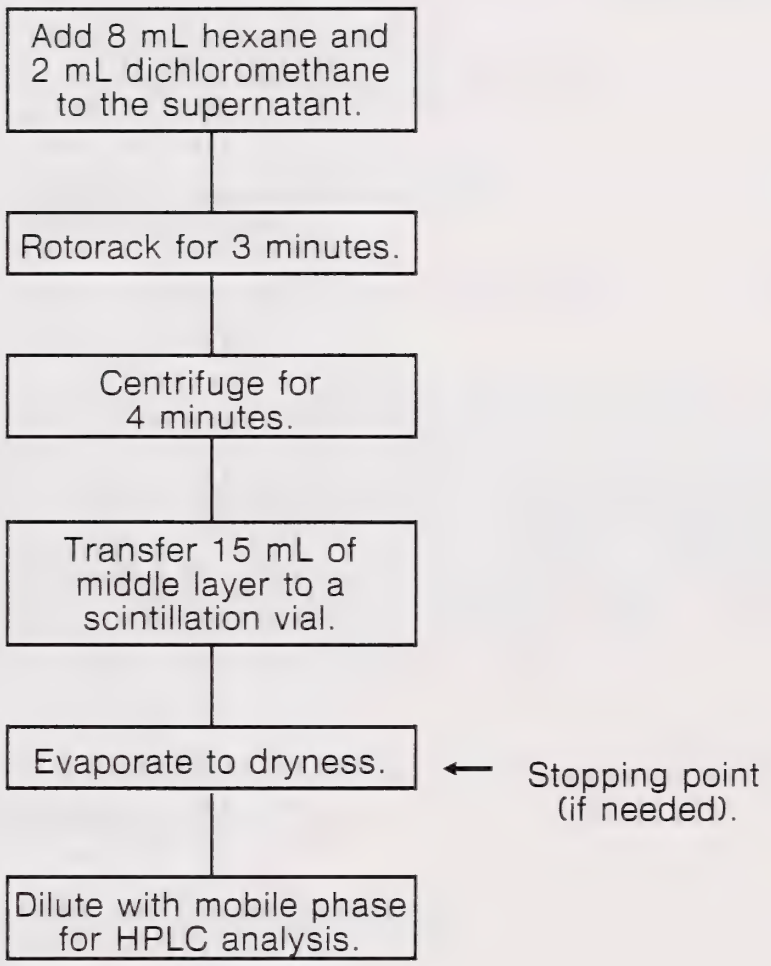
3. Flow Chart Summary



Continued on DEX-9.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTIFICATION

1. Instrumental Settings and Conditions

A schematic of the system is shown in Figure 1.

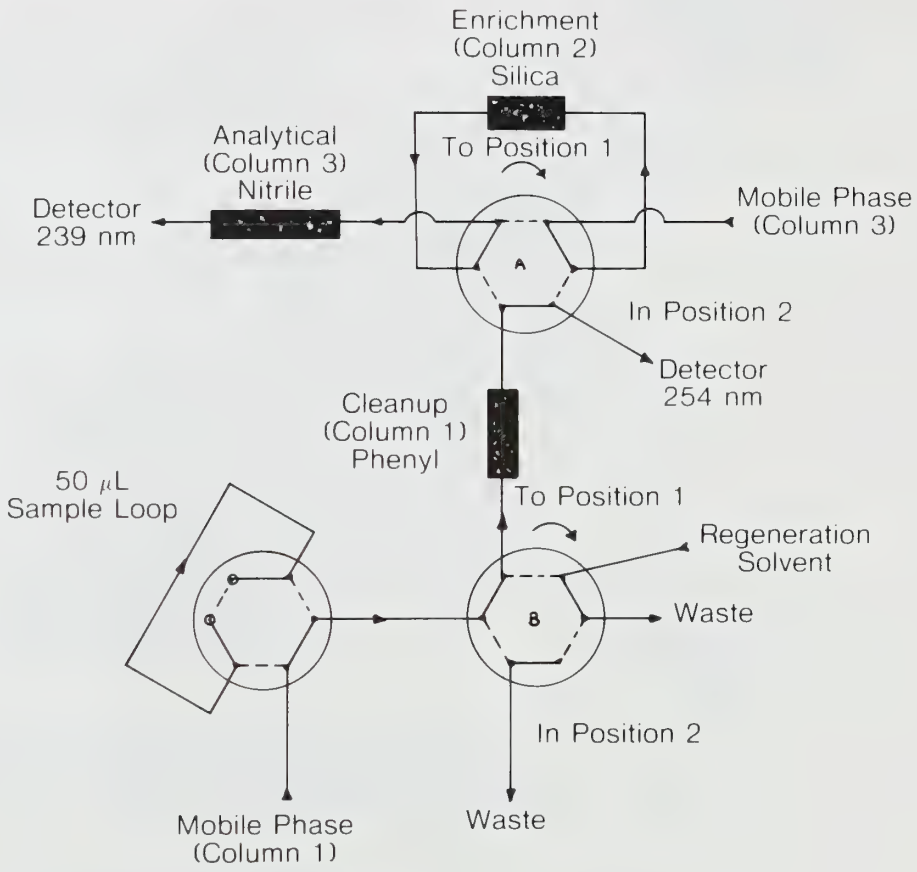


Figure 1

a. Column-Switching Timetable.

Time (Min)	*Valve A*	*Valve B*	*Event*
0	Position 2	Position 2	Injection.
3.2	Position 1		Begin collection of dex on column 2.
4.1	Position 2		End collection; begin integrator to monitor column 3 separation.
4.2		Position 1	Begin regeneration of column 1.
11.2		Position 2	End regeneration and begin equilibration of column 1.
15.2	Position 2	Position 2	End equilibration; ready for next injection.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTIFICATION (Continued)

b. HPLC Operating Conditions.

i. Mobile phases

(a) Column 1: 0.1% water, 0.1% acetic acid, 5.8% 2-propanol in hexane (% by volume).

(b) Column 3: 0.1% water, 0.1% acetic acid, 12.8% 2-propanol in hexane (% by volume).

(c) Regeneration solvent: 2% water, 49% methanol in dichloromethane (% by volume).

NOTE: Mobile phases were sparged with helium for several minutes before use. For example, with an Eppendorf pipet, 1 mL of acetic acid and 1 mL of water were added to the 100 mL graduated cylinder and brought to 60 mL with 2-propanol. This mixture was transferred to the 1-L graduated cylinder. The smaller cylinder was rinsed with hexane which is poured into the 1-L cylinder and the solution brought to 1 L with hexane. This method provided adequate batch to batch reproducibility, but volumetric flask may be preferred.

ii. Flow rate

1.5 mL/min for analysis on columns 1 and 3; 2.0 mL/min for regeneration of column 1.

iii. Attenuation

0.004 AUFS or as needed.

iv. Detection

Two absorbances detectors. If possible, monitor column 3 at 239 nm (lambda max for dexamethasone); 254 nm is acceptable for column 1.

v. Chart speed

1.0 cm/min

vi. Retention time

Approximately 3.5 minutes on column 1 and approximately 7.6 minutes on column 3. For any given day and batch of mobile phase, the reproducibility of these retention times should be within $\pm$ 0.5 minutes. After many days of sample injections, the retention time of dexamethasone on column 1 generally increases. To regain the original retention time, increase the percentage of 2-propanol in column 1 mobile phase.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTIFICATION (Continued)

2. HPLC Analysis

a. For HPLC use, prepare 0.2 ng/μL dexamethasone solution by diluting an aliquot of the methanol stock solution with column 1 mobile phase.

b. Inject 10 ng of dexamethasone (50 μL of above solution) to determine the retention time of dexamethasone on column 1.

NOTE: This is done at the start of each day of HPLC analysis and is checked approximately every two hours thereafter during an analysis.

c. Based on peak observed in step b, program collection window, allowing approximately 0.15 minutes on each side of peak base.

NOTE: The collection window was typically 0.9 minutes long.

d. Inject 10 ng dexamethasone and, using column-switching program, determine retention time on column 3.

NOTE: One detector monitors column 1 and the other monitors column 3. The appropriate detector must be connected to the recorder before each run. Column 1 is monitored for checking retention time of dexamethasone standard; column 3 is always monitored when samples are injected using switching program.

e. Prior to injection, dissolve liver and muscle residues in 500 μL of column 1 mobile phase respectively.

f. Cap vials and sonicate for 2 minutes.

g. Filter centrifugally, and transfer filtrate to auto sampler vials. Cap the autosampler vials if preparing many samples for an automated run.

h. Make duplicate injections of control and fortified tissues and record peak height to construct calibration curve. Before control tissue is injected, a blank (mobile phase) injection is made, though no ghosting was ever observed.

i. Make duplicate injections of each sample to be analyzed and record peak height.

j. For GC-MS confirmation, collect dexamethasone in 5 mL centrifuge tubes from approximately 0.4 minutes before to 0.4 minutes after its column 3 retention time. Immediately after collection, store tubes in freezer and perform derivatization no more than 24 hours later.

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Using data from the control (0 ppb) and fortified tissue samples, construct a least squares linear regression standard curve by plotting fortified tissue concentration against peak height (average from duplicate injection) for the resulting equation, $y = mx + b$.

   x = concentration (ppb) of dexamethasone in tissue

   y = dexamethasone peak height (average value from duplicate injections)

   m = slope

   b = y-intercept

b. Using the established regression line, determine the concentration of dexamethasone in each sample (x) from the average value peak height from duplicate injections (y).

   NOTE: For liver, a standard curve was constructed using tissue concentrations of 0, 20, 40, and 80 ppb. The correlation coefficient was typically 0.995.

   For muscle, a standard curve constructed using fortified tissue at concentrations of 0, 3, 5, and 10 ppb gave poor quantitative results. Quantification above 10 ppb may be possible but was not evaluated by this method.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Determination and Confirmation of Dexamethasone in Bovine Tissues

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat, fume hood.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Methanol Dichloromethane Acetonitrile Hexane Ethyl acetate 2-propanol	Organic solvents are flammable, irritating, and can cause toxic injuries.	Work in fume hood, away from any combustion devices.
Glacial acetic acid	Combustible, irritating, and can cause toxic injuries.	Work in fume hood, away from any combustion devices.
B-Glucuronidase		Avoid contact and inhalation.

4. Disposal Procedures

Organic solvents	See above.	Place chlorinated and nonchlorinated solvents in separate labeled waste containers until disposal by in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Dexamethasone Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

DEXAMETHASONE ANALYSIS

Analyst: _____ Date Started: _____

Date Completed: _____

STANDARD CURVE DATA

Standard Concentration	Peak Area #1	Peak Area #2	Average Peak Area
0 ppb			
20 ppb			
40 ppb			
80 ppb			

LINEAR REGRESSION LINE

Slope, m =
Intercept, b =
Correlation Coeff., r =

Sample ID	Tissue Code	Sample Weight	Final Volume	ppb

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

This section is only applicable for beef liver matrix. Further study has to be done for the evaluation of muscle.

1. Performance Standard

Analyte	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability CV_o %*
Dexamethasone	20	80-130	≤ 10
	40	80-130	≤ 10
	80	90-110	≤ 10

NOTE: Data for acceptable recovery in muscle tissue is unavailable at the present time.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Tissue condition	Should be kept frozen until assayed.
b. Sample weight	5.0 ± 0.1 g
c. pH adjusted (using glacial acetic acid)	4.2-4.7
d. B-glucuronidase: volume added	100 µL ± 10 µL
e. Incubation temperature	37° C ± 5° C
f. Evaporation temperature	60° C ± 5° C
g. Evaporation condition	Sample must be evaporated to dryness; all acetonitrile must be removed.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Duplicate standard curve at each of the following concentration levels on 3 consecutive days:

Solution Concentration	*Tissue Concentration*
0 ng/µL	0 ppb
1 ng/µL	20 ppb
2 ng/µL	40 ppb
4 ng/µL	80 ppb

ii. Phase II: Self-fortified samples—Duplicate analyses on fortified samples at 0, 20, 40, and 80 ppb on 3 different days, using beef liver.

NOTE: Phase I and Phase II may be performed concurrently.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Phase III: Check samples for analyst accreditation.

(a) 6 samples submitted by the supervisor with the following specifications:

(i) Matrix—beef liver.

(ii) Fortification level in tissue must be 20-80 ppb.

(b) Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst fortified at 20-80 ppb.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability

a. Matrix: Beef liver.

b. Sample receipt size: Minimum of 25 g.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: To be determined.

ii. Condition: −20° C.

6. Sample Set

a. Screening method.

For screening purposes, a sample set can include a blank tissue, a blank tissue fortified at the middle range of the standard curve, and up to 8-12 samples per set.

b. Quantitation method.

To be used for any samples found positive from the screening method above. Each set must contain:

i. A blank tissue.

ii. A fortified standard curve including:

(a) 20 ppb.

(b) 40 ppb.

(c) 80 ppb.

NOTE: Separate curve is needed for different tissue.

iii. All samples found positive from the screening method.

7. Sensitivity

a. Lowest detectable level (LDL): 10 ppb.

b. Lowest reliable quantitation (LRQ): 20 ppb.

c. Minimum proficiency level (MPL): not determined.

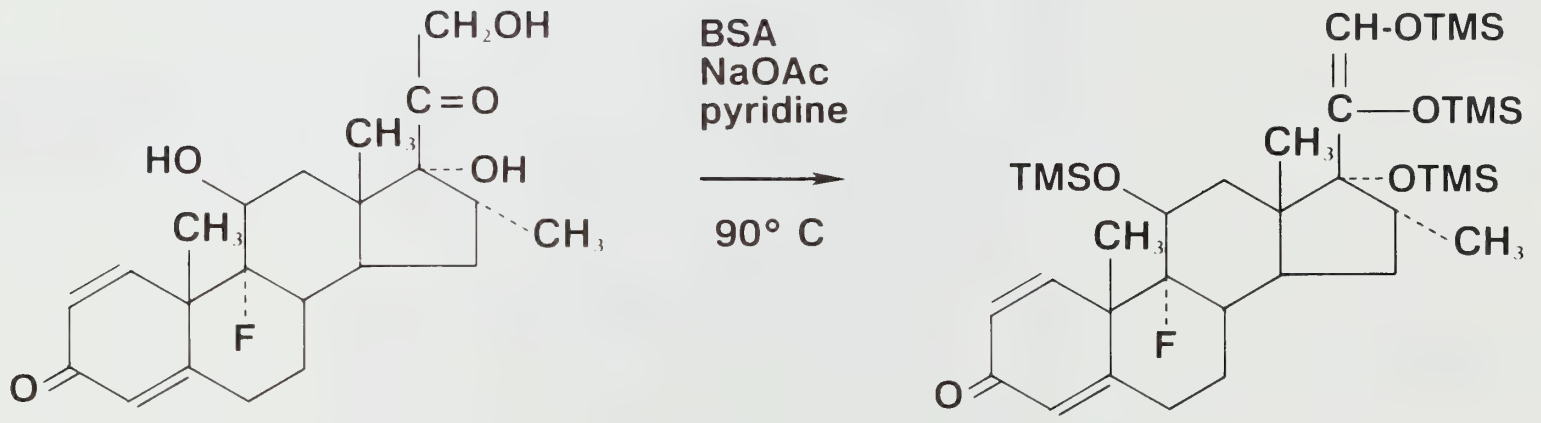
II. CONFIRMATORY METHOD

A. INTRODUCTION

Theory and Structure

The presence of dexamethasone in extracts of bovine liver and muscle obtained by the determinative method can be confirmed by using the HPLC-prepared extract converted to its TMS-enol-TMS derivative with BSA in the presence of sodium acetate and pyridine.

The derivative of dexamethasone is separated by capillary gas chroma-tography and four ions characteristic of it are monitored by electron ionization mass spectrometry in the selected ion monitoring (SIM) mode. Confirmation is based on retention time and presence of the four selected ions in the correct relative abundances.



Dexamethasone
MW = 392.20

Dexamethasone-TMS-enol-TMS
MW = 680.36
TMS = trimethylsilyl

II. CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted if necessary for all apparatus listed below:

a. Disposable glass centrifuge tubes, 5 mL, screw cap, with conical bottom—VWR Scientific.

b. Parafilm—American Can Co.

c. Syringes for volumetric additions, 10 μL and 100 μL—Hamilton Co.

d. Reacti-Therm Dry Block Heater—Pierce Chemical Co.

2. Instrumentation

a. Gas chromatograph—Carlo Erba Model 5160 Mega Series high resolution GC.

b. Mass spectrometer—Hewlett-Packard 5970 Series Mass Selective Detector with direct capillary interface.

c. Injector—Scientific Glass Engineering Model OC1-3 On-Column with On-Column Control Module (SGE, Austin, TX).

NOTE: Grob-type heated split-splitless injector may also be suitable but was not utilized in this work.

d. Syringe for GC injection—5 μL on column syringe.

e. Oxy-Purge nonindicating oxygen trap—Alltech Associates. Helium gas passes through trap before reaching GC column.

f. Capillary GC column—J&W Scientific DB-1, 0.25 mm $\times$ 15 m, 0.1 μm film thickness (Folsom, CA).

g. Data station—Hewlett-Packard 59970C Chem Station.

II. CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

All reagents listed below can be purchased from an alternate source if available (with equivalent specifications).

a. N, O-bis-(trimethylsilyl)-acetamide (BSA) in mL ampules—Pierce Chemical Co.

b. Acetone—ACS certified Fisher Scientific.

c. Pyridine—ACS certified Fisher Scientific.

d. Cyclohexane, hydrous sodium acetate ($CH_3COONa \bullet 3H_2O$).

e. Methanol—Fisher Scientific, HPLC grade.

f. Solution of hydrous sodium acetate in methanol, 1 $\mu g/\mu L$.

D. STANDARDS

See Determinative Method, section D.

II. CONFIRMATORY METHOD

E. DERIVATIZATION

1. Procedure

NOTE: Derivatization should be performed within 24 hours of HPLC sample collection in the Determinative Method.

a. Evaporate the HPLC samples collected in conical centrifuge tubes to dryness under a gentle stream of nitrogen at 60° C.

b. For TMS-enol-TMS derivative of dexamethasone standard, place 100 ng (2.55×10^{-10} moles) of dexamethasone (100 μL of methanol solution) in conical centrifuge tube and evaporate to dryness along with HPLC samples as in step a above.

c. Add 10 μL sodium acetate/methanol solution to each tube, vortex-mix for 10 seconds, and evaporate to dryness as in step a above.

d. Add 50 μL acetone to each tube, vortex-mix for 10 seconds, and heat in 90° C heater block for 100 minutes.

e. Add 5 μL BSA and 10 μL pyridine, cap vials, vortex-mix for 10 seconds, and heat in 90° C heater block for 100 minutes.

f. Allow tubes to cool to room temperature, then rinse condensation inside tubes with approximately 50 μL of cyclohexane and evaporate to dryness as in step a above.

g. Add 10 μL cyclohexane, vortex-mix for 10 seconds, and evaporate to dryness as in step a above.

h. Dissolve dexamethasone-TMS-enol-TMS standard in cyclohexane to obtain concentration desired for GC-MS injection. For instance, add 20 μL cyclohexane to obtain a concentration of 8.67 ng/μL, assuming the derivatization reaction produces 173.49 ng of dex-TMS-enol-TMS from 100 ng of dexamethasone in 100% yield.

i. Dissolve sample derivatives in approximately 100 μL cyclohexane for freezer storage.

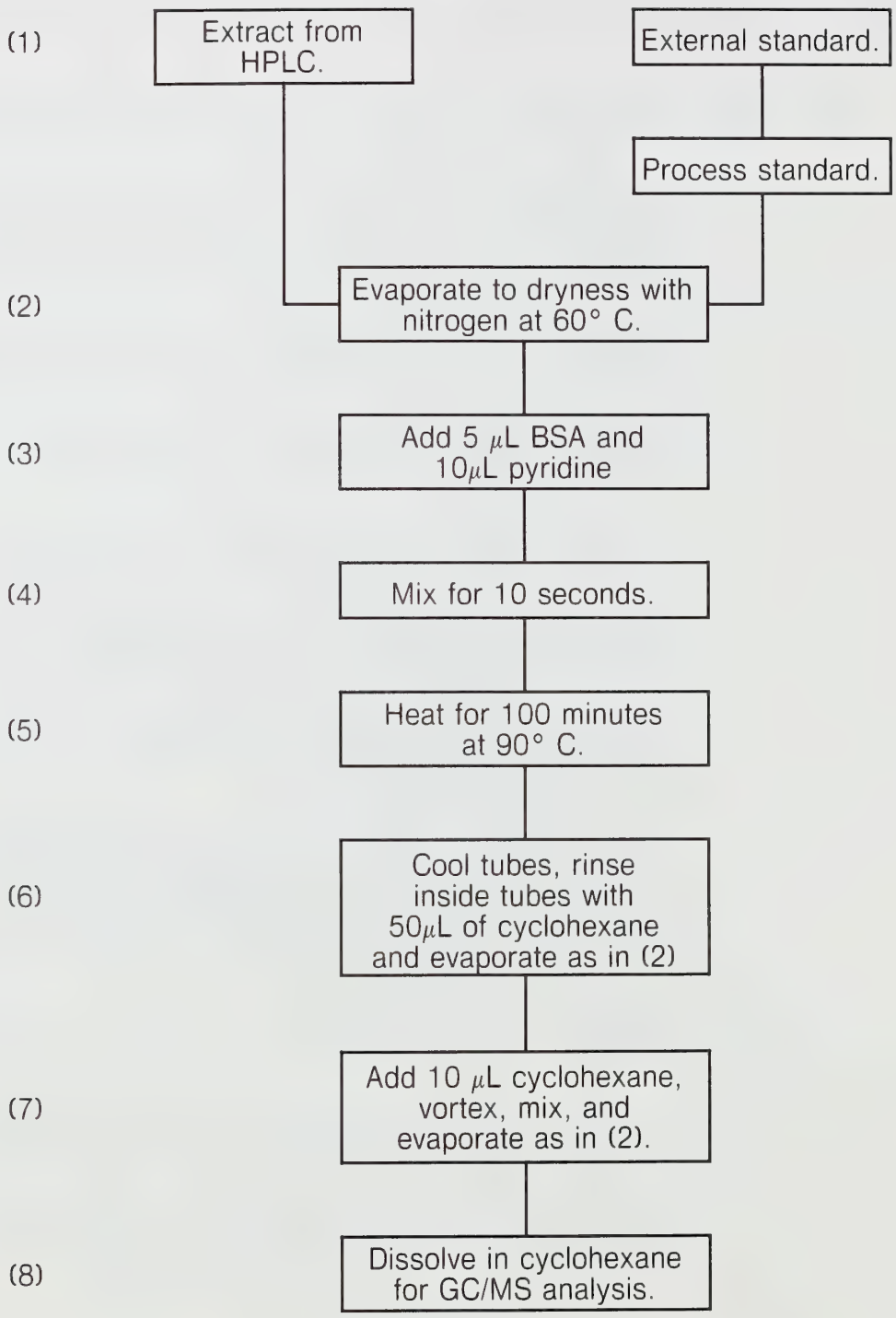
j. Seal caps with Parafilm and store vials in freezer (−10° C) until time of analysis. Derivative is stable under these conditions for at least a week.

k. Prior to GC-MS analysis, evaporate cyclohexane from sample derivatives and redissolve in 3 μL cyclohexane, all of which will be injected.

II. CONFIRMATORY METHOD

E. DERIVATIZATION (Continued)

2. Flow Chart Summary



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions.

Injection: on-column with oven at 60° C.

Oven temperature program: 60° C for 3 minutes, then programmed to 285° C at 40° C/min, held at 285° C until after dexamethasone-TMS-enol-TMS elutes.

Transfer line: 280° C.

Carrier gas: helium.

Flow rate (carrier gas): approximately 47 cm/second (measured at 285° C).

Retention time: 17.5 minutes (approximately).

b. MS conditions.

Set analyzer parameters with autotune program.

Adjust parameters necessary to meet decafluorotriphenylphosphine (DFTPP) specifications (15 ng in 1 μL ethyl acetate injected).

Electron energy: 70 eV.

Electron multiplier voltage: 200-400 V above autotune voltage.

Selected ion monitoring parameters: From 12-20 minutes, monitor the following four ions: 305.2, 332.3, 345.2, and 680.5, using a 100 millisecond dwell time for each ion.

2. GC/MS Analysis

a. Inject standard TMS-enol-TMS derivative in an amount comparable to amounts in suspect samples to be analyzed (i.e., within a factor of five). Make at least two injections; if time permits, more injections will provide better statistics for confirmation. For example, for confirmation in liver samples, 13 ng of dexamethasone-TMS-enol-TMS standard in 1.5 μL cyclohexane was injected twice.

NOTE: In determining amount of standard to inject, note that yield of TMS-enol-TMS derivative may vary for reaction of standard versus reaction in sample in matrix.

It is probably better to arrange for all standard and sample injection volumes to be the same.

b. Inject 3 μL cyclohexane to check for ghosting of dexamethasone-TMS-enol-TMS derivative, as well as column and solvent background. No ghosting was ever observed.

c. If control tissue samples are to be analyzed, inject them next, after the above solvent blank.

d. Inject samples dissolved in 3 μL cyclohexane.

e. Using integrated peak heights, calculate selected ion ratios relative to the molecular ion for standards and samples. Calculate averages of standard ratios.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

a. All four ions monitored must be present.

b. At least three of the four ratios must be within ± 20% of corresponding average ratio determined by injections of dexamethasone-TMS-enol-TMS standard at a level comparable to the estimated concentration of the sample.

c. The retention time must be within ± 20 seconds of the average retention time of dexamethasone-TMS-enol-TMS standard.

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Method Title — Determination and Confirmation of Dexamethasone in Bovine Tissues

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat, and fume hood.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
E. Derivatization		
Pyridine Acetone Methanol Cyclohexane	Organic solvents are flammable, irritating, and can cause toxic injuries.	Work in fume hood, away from any combustion devices.
BSA*	Combustible, irritating, a possible inhalation hazard, a possible mutagen, and carcinogen suggestive.	Work in fume hood, away from any combustion devices; avoid inhalation and contact. Self-contained organic vapor respirator suggested.

4. Disposal Procedures

Organic solvents	See above.	Place in labeled storage container until disposal by in-house specialist.
BSA*	Reacts somewhat with water and reacts rapidly with alcohol.	Avoid mixing with alcohol.

*BSA = N,O-bis-(trimethylsilyl)-acetamide

II. CONFIRMATORY METHOD

I. WORKSHEET

The worksheet on the facing page, *Mass Spectrometry Data Report*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte: _____ Tissue: _____

	R.T. (min) Base Peak	Ion Abundance (m/z)				Ion Ratios		
	_____	_____	_____	_____	_____	_____	_____	_____
Blank								
Standard								
Fortified Tissue								
Sample #								

Concentration:

Standard: _____

Fortified tissue: _____

Sample: _____

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb.

b. No false negatives at 20 ppb.

2. Critical Control Points and Specifications

Procedure Step	*Acceptable Control*
Derivatization of dexamethasone HPLC extract	Should be performed within 24 hours of HPLC sample collection
Volume BSA added	50 μL $\pm$ 1 μL
Volume pyridine added	10 μL $\pm$ 1 μL
Heating temperature	90° C $\pm$ 10° C
Heating time	100 minutes $\pm$ 10 minutes

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standards, show that the instrument is functioning properly.

ii. Phase II: Minimum of 3 samples for each type of matrix, with at least 2 positives fortified at 20 ppb.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: 3 check samples for analyst qualification meeting the same requirements as in 3.a.ii.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section F.4.

4. Intralaboratory Check Samples

Refer to Determinative Method, section J.4.

5. Sample Acceptability and Stability

Refer to Determinative Method, section J.5.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	Each set must contain a reagent blank, a blank tissue, a blank tissue fortified at 20 ppb, and all samples found positive from the quantification method.
7. Sensitivity	a. Lowest detectable level (LDL): 20 ppb. b. Lowest reliable confirmation (LRC): 20 ppb.

ETHYLENE DIBROMIDE

Contents

I. Determinative Method

A. Introduction 1

B. Equipment 2

C. Reagents and Solutions 3

D. Standards 4

E. Extraction Procedure 5

F. Analytical Quantitation 6

G. Calculations 7

H. Hazard Analysis 8

I. [Reserved]

J. Quality Assurance Plan 9

II. Confirmatory Method 11

I. DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Ethylene dibromide (EDB) has very low solubility in water and has a high vapor pressure, making codistillation over water with another solvent (hexane) a practical separation technique. The hexane-EDB condensate is trapped in a Barrett distilling receiver that is cooled in an ice bath. The water is drained from the receiver, the volume of hexane read, and the hexane layer transferred to a 20 mL scintillation vial containing 2-3 g Na_2SO_4. The dried condensate is injected directly on a gas chromatograph equipped with an electron capture detector.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus	a. Scintillation vial, 20 mL, Wheaton Scientific No. 98654, or equivalent. NOTE: Adhesive in the foil-lined cap of the vial may produce interfering peaks on GC. This can be prevented by adding a Teflon liner to the cap. b. Volumetric flasks, 100 mL and 10 mL. c. Syringes: 10 μL, 100 μL, and 1000 μL. d. Condenser water jacket, Kimax No. 18190 with 24/40 ground glass fitting, or equivalent. e. Barrett distillation receiver, 20 mL, Pyrex No. 3622, or equivalent. f. 1000 mL round-bottom flask with 24/40 ground glass fitting single neck. (Refer to note following item 1.g.) g. Heating mantle, 1 L, Glas-Cal Apparatus Company, 711 Hulman Street, Terre Haute, IN, 47803, catalog No. 0-408, or equivalent. NOTE: Flask size can be reduced to 500 mL and a hot water bath can be substituted for the heating mantle if these items are not available.
2. Instrumentation	Gas chromatograph, Hewlett-Packard 5880 (or equivalent) equipped with Electron Capture detector (^{63}Ni)

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane, UV grade, Burdick and Jackson, or equivalent.

b. Distilled water, Waters Milli Q treated, or equivalent.

c. Sodium sulfate, reagent grade (tested for interfering peaks), anhydrous crystals.

DETERMINATIVE METHOD

D. STANDARDS

1. Source	1,2-dibromoethane (> 99%), Aldrich Gold Label No. 24, 0065-6, b.p. 131-132° C.
2. Preparation of Standards	Prepare fresh working external and recovery studies once per month. Each fresh standard shall be prepared as stated in the methodology and compared to current standard. a. Stock standard (0.5 μg/mL): Add 23 μL of 2.179 specific gravity EDB to 100 mL volumetric flask and dilute to volume with hexane. b. Working standard (0.5 μg/mL): Add 100 μL of stock standard to a 100 mL volumetric flask and dilute to volume with hexane. c. External standard (1.0 ng/mL): Add 200 μL working standard to 100 mL volumetric flask and dilute to volume with hexane. d. Recovery standard: i. 1 ppb: Add 1 mL working standard to 500 mL hexane. ii. Add 10 mL recovery standard solution to 10 g blank tissue.
3. Storage Conditions	Store all standards in a freezer at 0° C.
4. Shelf Life Stability	Only long enough to conduct all necessary analyses—volatile compound.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

To prevent cross-contamination, sample is finely chopped on filter paper just before analysis.

2. Sample Extraction

a. Weigh 10 g of finely chopped sample in 1000 mL round-bottom flask and add 300 mL distilled water, 10 mL hexane, and boiling chips.

b. Connect to distillation apparatus and place in heating mantle.

c. Increase heat until water just boils and collect distillate until 2-3 mL water layer appears in Barrett trap.

d. Drain water layer and discard.

e. Add contents of trap (8.8 to 10.0 mL hexane) to 20 mL scintillation vial containing 2-3 g of anhydrous Na_2SO_4, shake vial, and let stand. When hexane is no longer cloudy, inject 5 μL on GC.

f. Prepare a reagent blank by distilling 10 mL hexane with 300 mL water and collect in the same manner as a sample.

g. Prepare a tissue blank by distilling 10 g of the tissue of interest with 10 mL hexane and 300 mL water, and collect in the same manner as a sample.

h. Perform recoveries at 1 ppb equivalent EDB by repeating steps a-e above. Repeat with 100 ppb EDB equivalent, if samples are found in this range. Use standards in section D.2.d.i, ii.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Instrumental Settings and Conditions

a. 6 ft × 2 mm glass column packed with 15% OV-17 on 80/100 mesh Chromosorb WAW or 20% OV-225/20% OV-17 (2 + 1) on 80/100 mesh Chromosorb W-HP.

b. Argon/methane carrier gas 95/5: 20 mL/min for 15% OV-17 column, 37 mL/min for 20% OV-225/20% OV-17 column.

c. Oven temperature programmed from 100° C for 8 min at 16° C/min to 220° C, hold for 8 to 10 min, time delay 15 min for equilibration. Injection temperature 160° C, detector temperature 350° C.

NOTE: Detector temperature can be maintained at 260° C for greater sensitivity during the analysis, but must be elevated to 350° C periodically to prevent contamination.

NOTE: Instrument should be adjusted to give approximately 50% full-scale response and a retention time of about 5-6 min for a 1 ppb external standard.

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure	Each chromatogram should contain injections of hexane, reagent blank, tissue blank, external standard (1 ng/mL), and 1 ppb recovery standard. Calculation is made against the external standard without correction for recovery.
2. References	Rains, D. and Holder, J., J. Assoc. Off. Anal. Chem., Vol. 64, pp 1252-1254, (1981)

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Analysis and Confirmation for Ethylene Dibromide in Animal Tissue by Codistillation.

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat, face mask.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
D. Standards		
1, 2-dibromoethane	This compound is flammable and extremely corrosive to the skin, eyes, and respiratory system. It is also considered to be a carcinogen.	Pipetting and diluting must be performed in an efficient fume hood. Electric hot plates or open flames should not be present.
E. Extraction	Vapor leaks, explosions	The procedure should be followed using ice baths and checking that the distillation components are sealed securely. It would be desirable for this procedure to be performed in a fume hood.

4. Disposal Procedures

Ethylene dibromide solutions	See above.	The solutions should be combined in a separate storage container until picked up by the contractor or in-house specialist.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppb)*	*Acceptable Recovery (%)*	*Repeatability CV (%)*
Ethylene dibromide	0.5-2.0	65%-100%†	15

†With average of 10 last recoveries not less than 75%.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Lot no, source, date checked, analyst.	No deflection > 0.05 ppb as EDB within ± 0.5 min of EDB R.T.

3. Readiness to Perform

a. Familiarization.

i. Phase I: Standards—3 levels, 3 replicates each.

(a) 0.5 ppb.

(b) 1.0 ppb.

(c) 2.0 ppb.

ii. Phase II: Fortified samples—at least 3 levels, 3 replicates each.

(a) 0.5 ppb.

(b) 1.0 ppb.

(c) 2.0 ppb.

Blanks: Reagent; Tissue: 1 of each with each set.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Three samples from FSIS Science Western Laboratory (or supervisor if only one laboratory is performing this test).

(b) Submit analytical findings to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section J. 1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Initially, 1 per set, gradually reduced to a minimum of 1 per week per analyst, or 20% of official samples analyzed (whichever is smaller). Blind samples or random duplicates chosen by the supervisor or LSO.

ii. Records to be maintained by analyst and reviewed by supervisor and LSO.

(a) Running average difference between replicates.

(b) All % recoveries recorded.

(d) For all recoveries the running average, standard deviation, and coefficient of variation.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Fat.

b. Sample receipt size: Varied; enough to obtain matrix required for all quantitative tests and reserve sample.

c. Condition upon receipt: Cold.

d. Sample storage:

i. Time: Maximum unknown—analyte dissipates.

ii. Condition: Frozen.

6. Sensitivity

a. Lowest detectable limit (LDL): 0.5 ppb.

b. Lowest reliable quantitation (LRQ): 1.0 ppb.

c. Minimum proficiency level (MPL): 1.0 ppb.

II. CONFIRMATORY METHOD

Confirmation of EDB Residue

a. Prior to injecting the samples, inject 5 μL of hexane to determine if there are any interfering peaks.

b. For EDB residue between 1 and 5 ppb, inject 5 μL of the 10 mL hexane distillate into the mass spectrometer, utilizing the following equipment and conditions:

   i. Gas chromatograph equipped with a 25 M OV-1 column. Injection port 150° C , column temperature 50° C. Use a splitless injection system for the analysis.

   ii. Operate mass spectrometer in the negative chemical ionization mode using methane as reagent gas. Tune the instrument on ion 633. Adjust the electronics for maximum response while just attaining separation between 633 and 634. Monitor ions 79 and 81. Interface temperature 200° C. Source temperature 200° C.

   iii. Minimum detectable amount should be between 1 and 5 pg of EDB when monitoring ions 79 and 81, with a retention time of 4.5 min.

c. For EDB residues between 5 and 20 ppb, concentrate the 10 mL hexane distillate to 1 mL at room temperature under a stream of nitrogen. Inject 5 μL of this concentrate into the mass spectrometer using the same GC conditions shown in procedure b above and the following MS conditions:

   i. Operate mass spectrometer in the electron impact mode. Perform a normal tune procedure using PFTBA as standard. Source and interface remain at 200° C. Monitor ions 107 and 109.

   ii. Minimum detectable amount should be less than the 250 pg injected.

d. A positive confirmation is reported if in procedure b both ions, 79 and 81, are present at the correct retention time and the ratio of 79 and 81 is approximately 1.00 $\pm$ 10%, and in procedure c if both ions, 102 and 109 are present at the correct retention time and the ratio of 107 and 109 is approximately 1.10 $\pm$ 10%.

FENTHION

Contents

A. Introduction 1
B. Equipment 2
C. Reagents 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 8
G. Calculations 9
H. Hazard Analysis 10
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

A weighed portion of tissue is ground in a mixture of acetone and chloroform and the aqueous phase is discarded. The organic phase is evaporated to dryness and the residue partitioned between petroleum ether and acetonitrile. The acetonitrile layer is evaporated to dryness and the residue is treated with an oxidation mixture and extracted with hydrochloric acid. The oxygen analog, sulfone, is extracted from the aqueous phase in chloroform and evaporated to dryness. The residue is dissolved in acetone and is quantitatively analyzed by means of a gas-liquid chromatograph using a KCl thermionic detector.

2. Applicability

This procedure is applicable to the determination of fenthion (0,0-Dimethyl 0-[4-(methylthio)-m-tolyl] phosphorothioate) and its metabolites in animal tissue.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus	a. Assorted laboratory glassware. b. Blenders: Waring or equivalent, equipped with one-quart jars. c. Centrifuge: Damon/IEC model BE-SO, or equivalent. d. Food chopper: Hobart or equivalent.
2. Instrumentation	a. Gas chromatograph: equipped with a detector, or equivalent. b. Rotary vacuum evaporator: all glass.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Acetone: Reagent, ACS, redistilled.

b. Acetonitrile: Technical, redistilled.

c. Chloroform: Reagent, ACS, redistilled.

d. m-Chloroperbenzoic acid, F.M.C. Corp., Villa Park, IL.

e. m-Chloroperbenzoic acid reagent: 10% (w/v) in isopropyl ether. Observe warnings on container label. Prepare only the amount needed.

f. Hydrochloric acid: 2.0N.

g. Hyflo Super-Cel: Johns-Manville.

h. Isopropyl ether: Reagent, ACS, Fisher Cat. #E-141.

i. Petroleum ether: Redistilled.

j. Sodium hydroxide: Analytical reagent, 0.5N.

DETERMINATIVE METHOD

D. STANDARDS

1. Source	EPA Director Environmental Monitoring System Laboratory Office of Research & Development Cincinnati, OH 45268 513-569-7610 (use modem)
2. Preparation of Standards	Fenthion standard solution (5 μg/mL in acetone). a. Weigh 0.05 g fenthion standard into a clean 100 mL volumetric flask. Make to volume with reagent acetone and shake to mix. b. Transfer 1 mL of this solution to a clean 100 mL volumetric flask. Make to volume with reagent acetone and shake to mix. This flask contains 5 μg/mL of fenthion.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Grind the entire sample in a food chopper in the presence of an equal amount of dry ice.

b. Place the sample material in frozen storage overnight to allow the dry ice to sublime.

2. Sample Extraction—Fat Tissue

a. Weigh 25 g of the chopped sample into a blender jar.

b. Add 200 mL petroleum ether and blend for three minutes at high speed.

c. Filter, with suction, through a Whatman #42 filter paper covered with a ⅛-inch layer of Hyflo Super-Cel.

d. Transfer the filtrate to a 500 mL separatory funnel.

NOTE: If the filtrate becomes cloudy, warm the solution before the transfer is made.

e. Return the filter cake and filter paper to the blender jar.

f. Add 200 mL acetonitrile and blend for two minutes at high speed.

g. Filter, with suction, through a Whatman #42 filter paper into the same filter flask.

h. Transfer the filtrate to the separatory funnel containing the petroleum ether extract.

i. Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 500 mL separatory funnel containing 100 mL of Skellysolve B.

j. Shake the second separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a 1,000 mL round-bottom flask.

k. Repeat the extractions in steps i and j, using 200 mL portions of acetonitrile.

l. Evaporate the combined acetonitrile extracts (600 mL) to dryness on the evaporator at 40° C. Continue with step E.4.

3. Sample Extraction—Other Tissues

a. Weigh 50 g of the chopped sample into a blender jar.

b. Add 15 g of Hyflo Super-Cel and 200 mL acetone and blend for three minutes at high speed.

c. Filter, with suction, through Whatman #42 filter paper.

d. Transfer the filtrate to a 1,000 mL separatory funnel.

e. Return the filter cake and filter paper to the blender jar.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

f. Add 200 mL chloroform and blend at high speed for three minutes.

g. Filter, with suction, through a Whatman #42 filter paper covered with a ⅛-inch layer of Hyflo Super-Cel into the same flask.

h. Rinse the blender jar with 100 mL chloroform and add the rinsings to the dry filter cake.

i. Transfer the filtrate to the separatory funnel containing the acetone extract.

j. Shake the separatory funnel for 20 seconds, allow the layers to separate, and draw off the lower phase through a 32 cm Whatman #12 fluted filter paper into a 1,000 mL round-bottom flask.

k. Evaporate the sample to dryness on the rotary evaporator at 40° C.

l. Transfer the sample residue to a 500 mL separatory funnel with 200 mL petroleum ether.

m. Rinse the flask with 200 mL acetonitrile and add the rinsings to the separatory funnel containing the petroleum ether solution.

n. Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 500 mL separatory funnel containing 100 mL petroleum ether.

o. Shake the second separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a 100 mL round-bottom flask.

p. Repeat the extractions in steps n and o with two 100 mL portions of acetonitrile.

q. Evaporate the combined acetonitrile extracts (400 mL) to dryness on the evaporator at 40° C.

4. Oxidation—All Samples

Start an appropriate fenthion standard.

a. Dissolve the residue from the acetonitrile evaporation in 10 mL of the m-chloroperbenzoic acid reagent.

b. Allow the sample to stand at room temperature for 30 minutes with occasional swirling.

NOTE: Do not allow the sample to remain in contact with the concentrated oxidant for more than 45 minutes.

c. Add 10 mL isopropyl ether and transfer the sample to a 125 mL centrifuge separatory funnel.

d. Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 125 mL centrifuge separatory funnel containing 20 mL isopropyl ether.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

e. Add 80 mL of 2.0N hydrochloric acid to the first separatory funnel and shake for 30 seconds.

f. Shake the second separatory funnel for 30 seconds, if necessary; then centrifuge the sample for 5 minutes at 800 rpm.

g. Draw off the lower phase into a 500 mL separatory funnel.

h. Draw off the lower phase of the first 125 mL separatory funnel into the second 125 mL separatory funnel and repeat steps f and g.

i. Add 200 mL chloroform to the 500 mL separatory funnel containing the aqueous extracts (160 mL).

j. Shake the separatory funnel for 30 seconds, allow the layers to separate, and draw off the lower phase into a second 500 mL separatory funnel.

k. Repeat step j with 100 mL chloroform.

l. Add 100 mL of 0.5N sodium hydroxide to the combined chloroform extracts and shake the separatory funnel for 30 seconds.

m. Allow the layers to separate, and draw off the lower phase through a 32 cm Whatman #12 fluted filter paper into a 500 mL round-bottom flask.

n. Evaporate the sample to dryness on the evaporator at 40° C, removing any traces of chloroform with a gentle stream of air.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

Chromatographic conditions.

a. Column: 16 inch × 3 mm i.d. borosilicate glass column packed with 10% D.C. 200 and 0.2% QF-1, or equivalent, on 80-100 mesh Gas Chrom Q.

b. Carrier gas: Nitrogen, 35 mL/min, 40 psi.

c. Hydrogen flow: 35 mL/min, 12 psi.

d. Air flow: 425 mL/min, 30 psi.

e. Temperature:

i. Column—210° C.

ii. Injection port—225° C.

iii. Detector—240° C.

f. Recorder chart speed: ½-inch per minute.

g. Electrometer range: 100.

h. Attenuation: 1.

2. General Operation

a. Dissolve the residue from oxidation in 2 mL acetone.

b. Using a microliter syringe, inject 4 μL of the sample or standard solution into the gas chromatograph.

c. Identify the fenthion peak by its retention time and measure the area produced on the recorder strip chart with a polar planimeter. At the operating conditions employed, the retention time for the fenthion oxygen analog sulfone is 3.5 minutes.

3. Reference

Anderson, R. J., et. al., *Journal of Agriculture and Food Chemistry*. 14, 6, 619 (1961).

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Calculation of the ppm of fenthion in a sample is done by use of the following equation in which response for an unknown is compared to the response for an amount of standard fenthion carried through the procedure from the oxidation step. In the case of a 50 g sample, 5 μg of standard fenthion is used, which corresponds to 0.1 ppm.

$$\text{ppm} = \frac{\frac{\text{Area}}{\text{(Sample)}}}{\frac{\text{Area}}{\text{(Standard)}}} \times \frac{\frac{\text{Attenuation}}{\text{(Sample)}}}{\frac{\text{Attenuation}}{\text{(Standard)}}} \times \frac{\text{Std. inj.}}{\text{Sample wt. in g}} \times \frac{\text{Dilution}}{\text{Factor}}$$

When using the aliquots and dilutions described in the above procedure, the equation simplifies to:

$$\text{ppm} = \frac{\frac{\text{Area}}{\text{(Sample)}}}{\frac{\text{Area}}{\text{(Standard)}}} \times \frac{\frac{\text{Attenuation}}{\text{(Sample)}}}{\frac{\text{Attenuation}}{\text{(Standard)}}} \times 0.1$$

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Fenthion Residues in Animal Tissues		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Acetone Acetonitrile Chloroform m-Chloroperbenzoic acid	These reagents are very flammable and corrosive, and the vapors are extremely irritating to the skin, eyes, and respiratory system.	These solvents should only be used in an efficient fume hood, away from any electrical heating devices.
	D. Extraction Procedure		
	Extraction of fat tissue Extraction of other tissues	The relatively large volumes of reagents listed above plus petroleum ether present an increased risk. The concentration of these solvents also poses a danger (rotary evaporation).	It is important that these steps be performed in the fume hood.
4. Disposal Procedures	Organic solvents	See above	Segregate chlorinated from nonchlorinated solvents to the extent possible. Hold in designated storage cans until disposed of by the contractor or in-house specialist.

GASOLINE

Contents

A. Introduction 1
B. Equipment 2
C. Reagents 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 6
G. [Reserved]
H. Hazard Analysis 7
I. [Reserved]
J. Quality Assurance Plan 8

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The canned product is cooled in a freezer before opening, because canned meats do not usually have enough headspace for conveniently taking a saturated vapor sample. Fat is transferred to a 10 mL glass vial, which is then sealed with aluminum foil and capped with a Teflon-coated rubber septum and aluminum cover. The vial is heated at 100° C for 15 minutes and a 100 μL injection is made using a gas-tight syringe on a gas chromatograph equipped with a flame ionization detector (FID).

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Nitrogen, hydrogen, and compressed air sources: At 20 mL/min (or 80 mL/min, if glass column is used), 10 mL/min, and 100 mL/min respectively.

b. Glass vials: 10 mL, with suitable septums and covers.

c. Gas-tight syringe: 100 μL, Hamilton (1710-N), or equivalent.

d. Syringes: 10 μL and 100 μL.

e. Forced draft oven, or other heating device: Set at 100° C.

f. Volumetric flask: 10 mL.

2. Instrumentation

a. Gas chromatograph: Hewlett-Packard Model 5830, or equivalent, equipped with a flame ionization detector.

b. Stainless steel column: 6′ × 4 mm i.d. glass column (80 mL/min N_2 flow rate), packed with G.P. 5% SP-1200/1.75% Benton 34, on 100/120 mesh Supelcoport.

DETERMINATIVE METHOD

C. REAGENTS

Reagent List

a. Gasoline: regular or unleaded.

b. Petroleum ether: pesticide grade.

c. Ortho xylene: reagent grade.

d. Meta xylene: reagent grade.

e. Para xylene: reagent grade.

f. Ethyl benzene: reagent grade.

DETERMINATIVE METHOD

D. STANDARDS

1. Source	Gasoline: purchase from local source, regular or unleaded. Each new lot of standard should be prepared as stated in the methodology and compared to current standards.
2. Preparation of Standards	Working standards. i. Gasoline—1:10, gasoline/petroleum ether, V/V. ii. Ortho xylene—1:10, o xylene/petroleum ether, V/V. iii. Meta xylene—1:10, m xylene/petroleum ether, V/V. iv. Para xylene—1:10, p xylene/petroleum ether, V/V. v. Ethyl benzene—1:10, ethyl benzene/petroleum ether, V/V.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Place canned meat sample in a freezer for one hour.

2. Sample Extraction

a. Transfer approximately 1 g fat from the surface of the solidified meat mass inside the can to a 10 mL glass vial.

b. Seal the vial with aluminum foil, a Teflon-coated rubber septum, and the aluminum cover cap, in that order.

c. Run 1 ppm and 10 ppm standards (approximations only) in parallel with sample by adding 10 μL and 100 μL respectively of 1:10 gasoline/petroleum ether to 1.0 g clean fat in each of two vials and performing steps d and e below.

d. Heat the sample in the sealed vial in a forced draft oven at 100° C for 15 minutes. If heating block with holes that fit vials is substituted for oven, sample can be taken for step e below while vials remain at 100° C.

e. Using a 100 μL gas-tight syringe, inject a 100 μL headspace sample immediately from the heated vial into a GC equipped with one of the two columns described in described in B.2.b under the following conditions:

i. Injection temperature: 100° C.

ii. Column temperature: 75° C.

iii. FID temperature: 200° C.

iv. Nitrogen carrier gas flow rate: 20 mL/min (stainless steel, 6′ × 1/8″ i.d. column) or 80 mL/min (glass, 6′ × 4 mm i.d. column).

v. Hydrogen flow rate: 10 mL/min.

vi. Compressed air flow rate: 100 mL/min.

The best technique for injection is by ''pumping'' the syringe six times at moderate speed, filling the syringe slowly and making the injection within 30-90 seconds after the removal of the vial from the oven. Delay of injection results in drastically reduced response.

Retention time for all lower boiling fractions is approximately 25 minutes. Do not perform next injection until these fractions are eluted.

f. Inject 10 μL each of the o, m, and p xylenes, and the ethyl benzene working standards.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Identification	Quantitation for gasoline is not possible because instrument response is not linear. Repeatability with the same tissue is good. Positive identification is made by comparing the retention times and relative peak heights of o, m, and p xylenes and ethyl benzene. The lower detection limit depends upon the sensitivity of the FID, but will be in the vicinity of 0.1 ppm gasoline.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Detection of Gasoline in Canned Meat by GC Headspace Analysis		
2. Required Protective Equipment	Safety glasses, heat-resistant gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Gasoline Petroleum ether Ortho xylene Meta xylene Para xylene Ethyl benzene	These reagents are very flammable and corrosive and the vapors are extremely irritating to the skin, eyes, and respiratory system.	These solvents should only be used in an efficient fume hood, away from any heat-generating devices.
4. Disposal Procedures	Organic solvents	See above	Segregate chlorinated from nonchlorinated and hold in designated storage cans until disposed of by the contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Because this is a semiquantitative procedure, normal performance standards do not apply.

Method sensitivity: ≥ 1 ppm.

2. Critical Control Points and Specifications

Because the analytical procedure is only for the detection of the presence of gasoline, there are no truly critical control points. However, there are specifications in technique.

Record	*Acceptable Control*
a. Vial sealing	First—aluminum foil; second—Teflon-coated rubber septum; third—aluminum cover cap.
b. GC injections	Inject immediately after completing step E.2.d. Use pumping technique described in written procedure. Do not perform next injection until all lower boiling fractions have eluted.

3. Readiness to Perform

a. Familiarization

i. Phase I: Standards—By at least 3 replicate injections of standard, show that instrument is functioning properly.

ii. Phase II: Minimum of three samples, with at least 2 positives.

Submit data on standards and samples to Chemistry Division for information.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples submitted by supervisor.

(b) Report data to Chemistry Division, QSB.

b. Acceptability criteria.

No false positives or negatives.

NOTE: This section is only applicable if GAS is included in the regulatory monitoring sampling plan. For Emergency Response, a sufficient number of positive and negative controls are used to qualify the data set rather than the analyst.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample per analyst per set of samples.

ii. Random replicates chosen by supervisor or Laboratory QA Officer.

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QC Officer for all replicate findings.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: As required by exposure incident.

b. Sample receipt size: Varied; enough to accomodate all required testing.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: Maximum unknown; analyte dissipates from matrices.

ii. Condition: Frozen.

GENTAMICIN IN PORCINE TISSUE BY HPLC

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 10
G. [Reserved]
H. [Reserved]
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This is a sensitive, specific, reproducible HPLC method for the qualitative determination of gentamicin in porcine tissue. Concentrations as low as 0.4 ppm can be determined without interference from other chemical substances. Tissues are extracted with 1N sulfuric acid and cleaned up using column chromatography and liquid-liquid solvent extraction, followed by Sep-Pak® chromatography. The identification of gentamicin is obtained by post column, isocratic HPLC using a retention time standard (netilmicin).

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Blender, commercial dual-speed model: Waring, or equivalent.

b. Centrifuge: Sorvall RC-5B, fitted with model SS-34 rotor; DuPont Instruments, or equivalent.

c. Centrifuge: Damon UV-IEC; IEC Division, International Equipment Company, or equivalent.

d. Centrifuge tubes: 10 mL conical, graduated; Pyrex® , or equivalent.

e. Centrifuge tubes: 50 mL conical, graduated; Pyrex® , or equivalent.

f. Centrifuge tubes: 50 mL plastic; Sorvall by DuPont, or equivalent.

g. Fluorometer: Kratos model FS970, or equivalent, equipped with:

   i. 5 μL flow cell.

   ii. Overload reset—FSA986.

   iii. Computer interface—FSA987.

h. Glass chromatographic column (id = 10.5 mm, length = 20 cm), fitted with Teflon stopcock; SGA Scientific, Inc., or equivalent.

i. Glass wool: Pyrex wool filtering fiber; Corning Glass Works, or equivalent.

j. Balance, Analytical: Mettler model H-51; Fisher Scientific Co., or equivalent.

k. Balance, Top-loading: Mettler model P1200N; Fisher Scientific Co., or equivalent.

l. pH meter: Equipped with Sensorex SG900C combination electrode; Corning Scientific Instruments, or equivalent.

m. Pipetman: Adjustable digital microliter pipets (P20, P200, P1000); Gilson, or equivalent.

n. Plastipak syringes: 20 cc; Becton, Dickinson and Co., or equivalent.

o. PRP-1-HPLC analytical column (id = 4.1 mm, length = 150 mm, particle size = 10 μm); Hamilton Co., or equivalent.

p. Polytron: Willems, Brinkmann Instruments, or equivalent, equipped with:

   i. Homogenizer—model PT 10-35 basic assembly complete.

   ii. Generator—model PT 20-ST (sawtooth).

q. Reaction coil: Stainless steel tubing (1.6 mm O.D. × 0.23 mm I.D. × 1.5 m long); Waters Associates, or equivalent.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

r. Solvent clarification kit: Waters Associates, or equivalent, consisting of:

i. Glass filter apparatus.

ii. Filters for aqueous solvents: 0.45 μm (catalog #85117).

s. Stirrer, magnetic: Versamix; Fisher Scientific Co., or equivalent.

t. Tee, low dead volume mixing: #1-1HBZ; Parker Co., or equivalent;

u. Thermomix: Model 1419, heating and circulating unit with 5 L water bath; B. Braun or equivalent.

v. Vortex: Model K-55-G; Fisher Scientific Co., or equivalent.

2. Instrumentation

High-pressure liquid chromatograph system: HPLC, Waters Associates. Comprised of the following components coupled via the Interlink® System:

a. System controller: Model 720.

b. Data module: Model 730.

c. Solvent delivery system: Model 6000A.

d. Intelligent sample processor (WISP): Model 710B.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

All reagents/chemicals are of analytical grade.

a. Acetic acid.

b. Ammonium hydroxide.

c. Chloroform, distilled in glass: Burdick & Jackson, or equivalent.

d. IRC-50 (Amberlite®) cation exchange resin, chloride form, 20-50 mesh: Mallinckrodt, or equivalent.

e. 1-Pentane sulfonic acid, sodium salt: Eastman Kodak Co., or equivalent.

f. Methyl alcohol, distilled in glass: Burdick & Jackson, or equivalent.

g. 2-Mercaptoethanol, 98%: Aldrich Chemical Co., or equivalent.

h. Milli-Q water from water purification system: Millipore Corporation.

i. o-Phthalaldehyde (OPT), 98%: Alfa Products, or equivalent.

j. Potassium tetraborate powder ($K_2B_4O_7 4H_2O$).

k. Sep-Pak® (C_{18}): Waters Associates.

l. Sodium sulfate, anhydrous.

m. Sodium hydroxide pellets.

n. Sulfuric acid.

o. Tetrabutylammonium hydroxide: 40 wt. % aqueous solution; Aldrich Chemical Co., or equivalent.

p. HPLC mobile phase (prepare daily).

   i. Combine 300 mL methanol with 700 mL water.

   ii. Add 6.5 mL of tetrabutylammonium hydroxide solution.

   iii. Stir with a stir bar on a magnetic stir plate for 3 min.

   iv. Assemble solvent clarification kit as shown in the manufacturer's instructions, using an aqueous filter (0.45 μm) between the funnel and filter support screen.

   v. Immediately prior to use, filter the solution through the solvent clarification apparatus, under vacuum, while stirring the filtrate with a stir bar on the magnetic stirrer. Mobile phase is ready for use.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

q. o-Phthalaldehyde reagent (OPT) (prepare daily).

i. Weigh 60 mg of o-phthalaldehyde (OPT).

ii. Add 1 mL methanol.

iii. Mix solution with vortex mixer until OPT is dissolved.

iv. Add 0.2 mL 2-mercaptoethanol to the OPT solution and mix on a vortex mixer.

v. Add the resultant solution to 100 mL of 0.4M aqueous potassium tetraborate solution.

vi. Mix with a stir bar in the magnetic stirrer for 2 min.

vii. Filter the resultant solution through the solvent clarification assembly with a 0.45 μm filter under vacuum while stirring with a stir bar on the magnetic stirrer.

viii. Protect this reagent solution from light by wrapping the container with tin foil and cover the OPT reagent with a blanket layer of nitrogen. Reagent is now ready for use.

r. Ion-exchange resin (Amberlite® —IRC-50—ammonia phase).

i. Add 75 g of IRC-50 (Amberlite®) resin to 300 mL of 2N ammonium hydroxide in a 500 mL Erlenmeyer flask.

ii. Mix by swirling stoppered flask for 1 min. Let stand overnight at room temperature before use. This resin slurry may be stored up to 2 weeks in a stoppered flask at room temperature.

s. Sep-Pak® elution solvent.

i. Add 2 mL methanol to 98 mL water.

ii. Weigh 7.1 g of anhydrous sodium sulfate.

iii. Weigh 0.95 g of 1-pentane sulfonic acid sodium salt.

iv. Measure 1 mL acetic acid, using a 1 mL volumetric pipette.

v. Combine reagents i through iv and stir to dissolve the solids, using a magnetic stirrer.

vi. Assemble solvent clarification kit, using an aqueous filter (0.45 μm).

vii. Filter solution through solvent clarification assembly while stirring with the magnetic stirrer under vacuum. Reagent is ready for use (use within 6 weeks).

DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Gentamicin C components (C_1, C_{1a}, C_2): Compound Distribution, Corporate Research Div., Schering Corporation.

b. Gentocin® : Gentamicin sulfate veterinary solution (50 mg gentamicin base/mL); Schering Corporation.

c. Netilmicin sulfate (Sch 20569): Schering Corporation.

2. Preparation of Standards

a. Gentocin® standard solution: A 100 μg/mL base equivalent standard is prepared by adding 20 μL Gentocin solution (batch #OKMF115 P64883) to a 10 mL volumetric flaks and adding water to volume.

b. Gentamicin C component standard solutions.

i. C_{1a} sulfate (Sch 13707, batch 7017-119-I). A 100 μg/mL base equivalent standard is prepared in water.

ii. C_2 sulfate (Sch 13705, batch #7017-122-I). A 100 μg/mL base equivalent standard is prepared in water.

iii. C_1 sulfate (Sch 13706, batch #7017-92-II). A 100 μg/mL base equivalent standard is prepared in water.

c. Netilmicin standard and working standard solution.

i. A 1 mg/mL base equivalent standard solution is prepared by adding 17.15 mg of netilmicin sulfate (batch #NIM-O-N-40, potency 583 μg/mg base equivalents) to a 10 mL volumetric flask and adding water to volume.

ii. 1 mL of a 1 mg/mL base equivalent solution is placed in a 10 mL volumetric flask and brought to volume with water to give a 100 μg/mL base equivalent working standard.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Immediately prior to use, thaw tissue homogenate and weigh 10g into a plastic 50 mL Sorvall centrifuge tube.

b. Run a fortified and blank tissue with each set of samples. For a fortified tissue, add 40 μL of the Gentocin standard solution (100 μg/mL) to the thawed homogenate, using a Pipetman (P-200) syringe.

c. Add 12 mL 1N H_2SO_4 to the thawed homogenate.

d. Homogenize tissue mixture for 30 sec using the Polytron at low-speed setting.

e. Centrifuge the resulting homogenate for 10 min at 20,000 rpm in a Sorvall RC-5B centrifuge.

f. Decant upper aqueous solution (supernatant) into a glass beaker.

g. Add 10 mL water to the tissue pellet remaining in the tube.

h. Homogenize for 30 sec at low speed, using the Polytron.

i. Centrifuge for 10 min at 20,000 rpm in a Sorvall RC-5B centrifuge.

j. Decant the supernatant and combine with supernatant from step f.

k. Using the pH meter, adjust combined supernatant to pH 4.0 with 1N and/or 10N NaOH solution.

l. Centrifuge the resultant solution at 20,000 rpm for 10 min.

m. Decant the supernatant fluid.

n. The extract sample is now ready for the IRC-50 ammonia phase column.

2. Preparation of IRC-50 (Ammonia Phase) Column

a. Place a glass wool plug into the bottom of a 20 cm glass column.

b. Fill the column to give 10 mL bed volume with IRC-50-ammonia phase resin (refer to section C, Reagent and Solution List, item r).

c. Wash with 60 mL of water at 4 mL/min through column and discard the aqueous elute. Do not allow the column to run dry.

d. Place glass wool plug on top of column head.

e. Column is now ready for use.

3. Column Chromatography of Tissue Extracts

a. Place the tissue extract (1.n) onto the top of the IRC-50 (ammonia phase) column (2.e).

b. Adjust the flow rate of the column eluate to 4 mL/min. Allow column to run until about 1 mm of liquid remains on top of upper glass wool plug on the column.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

c. Discard aqueous eluate.

d. Eluate column with 5 mL of water.

e. Discard eluate.

f. Eluate the column with 2 mL of 0.5N NH_4OH.

g. Discard eluate.

h. Elute column with 10 mL of 1.0N NH_4OH and collect the resultant eluate into a clean 50 mL glass conical graduated centrifuge tube.

i. The eluate is ready for the liquid-liquid solvent extraction purification step.

4. Purification of Tissue Extract by Liquid-Liquid Solvent Extraction

a. Add 10 mL chloroform to the column eluate (3.h).

b. Vortex mix for 15 sec.

c. Centrifuge the resultant mixture for 5 min at 1500 rpm with Damon IEC-UV centrifuge.

d. Pipette bottom chloroform layer and discard the chloroform.

e. Add 10 mL chloroform to remaining aqueous solution.

f. Vortex mix for 15 sec.

g. Centrifuge the resultant mixture for 5 min at 1500 rpm with Damon IEC-UV centrifuge.

h. Pipette bottom (chloroform) layer and discard the chloroform.

i. Dilute remaining aqueous solution to 45 mL with water.

j. Sample is now ready for Sep-Pak chromatography.

5. Sep-Pak® Chromatography

a. Pass 10 mL of methanol through the Sep-Pak C_{18} cartridge using a 20 mL syringe.

b. Discard eluate.

c. Pass 10 mL of water through in the same manner.

d. Discard eluate.

e. Fill the 20 mL syringe with the tissue extract (4.i).

f. Pass the tissue extract through the cartridge slowly (approximately 20 mL/min).

g. Discard the eluate.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

h. Repeat steps e-g until all the extract (45 mL) has been processed.

i. Pass 20 mL of water through the cartridge and discard eluate.

j. Pass 20 mL of methanol through the cartridge and discard eluate.

k. Pass 20 mL of water through the cartridge and discard eluate.

l. Pass 20 mL of air through the cartridge to remove any remaining liquid.

m. Using the elution solvent (refer to section C, Reagent and Solution List, item s) in the syringe, collect the initial 2 mL eluate from the cartridge in a 10 mL conical glass graduated tube.

n. Add 30 μL of a 100 μg/mL base equivalent aqueous solution of netilmicin.

Sample is now ready for HPLC analysis, and may be stored up to 48 hrs at ambient temperature.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. HPLC System

a. The individual components of the system are assembled using 1.6 mm od × 0.23 mm id stainless steel tubing. All systems are under the control of the Model 720 system controller except for the detector and the post column derivatization system.

b. The HPLC system operation programming uses the appropriate standardized procedure as detailed in the Waters Associates, Inc., Data Module (No. IM82908, November 1979, Rev.8), System Controller (No. 82477, March 1980) and WISP (No. IM83697, April 1980) instruction manuals.

c. Operation of the fluorometric detector and accessories is in accordance with Kratos Schoeffel Instrument, FS970 L.C. Fluorometer instruction/ maintenance manual (11.22.78). The output from the detector is fed via Kratos computer interface FSA987 to the Waters Data Module.

d. The post column derivatization system is comprised of a Waters Model 6000A pump, low dead volume mixing tee, and solvent reservoir containing o-phthalaldehyde reagent (refer to section C, Reagent and Solution List, item q). The reagent solution enters the mixing tee at 180 degrees to the column effluent and the mixed liquids exit the T, to the reaction coil, via the stem exit of the tee.

e. The reaction coil is connected directly to the fluorometer detector. The effluent from the detector is collected in a waste container (2 L Erlenmeyer flask).

2. HPLC System Operating Conditions

a. Mobile phase (refer to section C, Reagent and Solution List, item p) flow rate: 0.6 mL/min.

b. Sample volume injected: 50 μL.

c. Column (PRP-1 column) operated at 24° C by immersion in water bath (refer to section B.1, item u).

d. o-Phthalaldehyde reagent solution (refer to section C, Reagent and Solution List, item q) flow rate: 0.4 mL/min.

e. Reaction coil at ambient temperature.

f. Detector conditions: Excitation wavelength 335 nm, emission wavelength 418 nm, emission filter KV418, absorbance range 0.2 μA, suppression Hi, time constant 7 sec automatic overload reset (FSA986) 0.5 min.

g. Data module chart speed: 0.5 cm/min.

h. Sample run time: 20 min.

i. Void volume of system: 1.6 mL.

3. Reference

NADA 103-037 and 91-191, Schering.

HALOFUGINONE

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 11
G. Calculations 12
H. [Reserved]
I. Worksheet 13
J. Quality Assurance Plan 17

II. Confirmatory Method 20

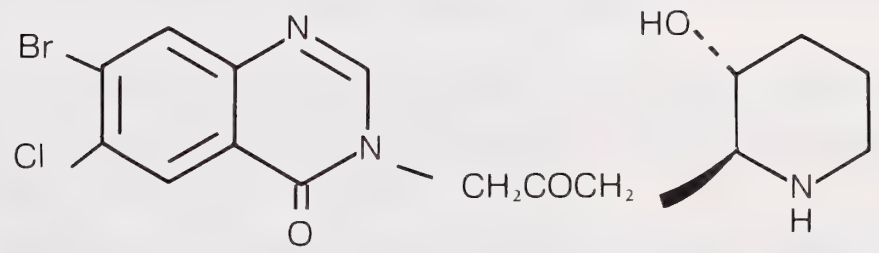
I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

a. Halofuginone is a coccidiostat for young chickens and young turkeys. In higher doses, halofuginone is a growth depressant, impairs feed utilization, and reduces feed intake. In rats it causes alopecia. The compound is prohibited from use during the last four days before slaughter (withdrawal period).

b. Halofuginone is extracted from enzyme-digested chicken liver as a free base into ethyl acetate and partitioned into ammonium acetate buffer solution. The halofuginone is concentrated with Sep-Pak C_{18} cartridges and eluted with methyl alcohol. The eluant is evaporated to dryness and the residue dissolved in the HPLC mobile phase. Analysis is achieved by HPLC using a BONDAPAK C_{18} column and a variable wavelength UV detector.



2. Applicability

This method is applicable for chicken and turkey liver.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Centrifuge, IEC, or equivalent, fitted with a rotating head capable of accepting 200 mL centrifuge bottle.

b. Food processor.

c. pH meter, Horizon Model 5998-10, or equivalent.

d. Single pan balance, Mettler P-1200, or equivalent.

e. Analytical balance, Mettler Gram-atic Type 6, or equivalent.

f. Water bath, Thelco Model 83, or equivalent.

g. Vortex mixer, Matheson Scientific Super-Mixer, or equivalent.

h. Evaporator, rotary.

i. General laboratory equipment and silanized glassware.

NOTE: Silanize the halofuginone-collecting glassware, the 15 mL test tubes, and the 250 mL round-bottom flasks.

2. Instrumentation

a. High-pressure liquid chromatograph system, Waters, or equivalent.

i. Waters Model 6000A solvent delivery system.

ii. Waters Intelligent Sample Processor (WISP), Model 710B.

iii. Kratos Spectroflow 773 variable wavelength dectector.

iv. Waters Data Module Model 730 integration system.

b. HPLC column, Waters μ-BONDAPAK C_{18}, 30 mm $\times$ 4 mm id, or equivalent.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solutions List

a. Ethyl acetate, Burdick & Jackson, distilled in glass, or equivalent.

b. Water, Milli-Q®, or equivalent.

c. Acetonitrile, Burdick & Jackson, distilled in glass, or equivalent.

d. Methanol, Burdick & Jackson, distilled in glass, or equivalent.

e. AquaSil, Pierce, water-soluble silanizing fluid.

f. Sodium chloride, Fisher Scientific analytical reagent, or equivalent.

g. Sodium carbonate, Fisher Scientific analytical reagent, or equivalent.

h. Sodium carbonate solution (10% w/v). Dissolve sodium carbonate (100 g) in water and dilute to 1 L in a volumetric flask.

i. Salt-saturated sodium carbonate solution (5% w/v). Dissolve sodium carbonate (50 g) in water and dilute to about 750 mL in a 1 L volumetric flask. Add sodium chloride, mixing, until the solution is saturated. Bring to volume.

j. Glacial acetic acid, Fisher Scientific, reagent grade, or equivalent.

k. Ammonium acetate, HPLC grade.

l. Ammonium acetate buffer (0.25M; pH 4.3). Add ammonium acetate (19.27 g) and acetic acid (30 mL) in water to 1 L volumetric flask. Dilute to volume with water.

m. Ammonium acetate buffer (0.125M; pH 4.3). Dilute 500 mL of 0.25M ammonium acetate buffer to 1 L with water in a volumetric flask.

n. Trypsin, Fisher Scientific, or equivalent.

o. Filter paper, Whatman GF/F, or equivalent.

p. Filter units, Millex®, HV 0.45 μm filter unit.

q. Sep-Pak C_{18} cartridge.

s. Preparation of Sep-Pak C_{18} cartridge.

   i. In preparation of HPLC analysis, wash cartridges sequentially with 2 mL methanol and 5 mL water.

   ii. In preparation for MS analysis, wash cartridges sequentially with 5 mL methanol and 10 mL of water.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Halofuginone hydrobromide (halofuginone).

Hoechst-Roussel Agri-Vet Company
Manager, Nutritional Research
P.O. Box 2500
Somerville, NJ 08876-1258
Telephone: 205/231-2000

2. Preparation of Standards

a. Stock standard.

Weigh 50 mg of halofuginone into a 250 mL volumetric flask. Dilute to volume with 0.25M ammonium acetate buffer (200 μg/mL).

b. HPLC calibration standards.

mL Used from Stock Standard	*Final Volume (mL)*	*Concentration (ppm)**
0.0	100	0
0.25	100	0.05
0.50	100	0.10
1.0	100	0.20

*Concentration calculated based on 20 g of sample.

c. Fortification standard.

Pipet 1 mL of stock standard into a 100 mL volumetric flask and dilute to volume with distilled water (2 μg/mL).

3. Storage Conditions

All standards are to be refrigerated at 4° C in tight glass bottles.

4. Shelf Life Stability

a. Stock standards: Replace every three months.

b. HPLC calibration standard: Replace every three months.

c. Fortification standard: Replace every month.

NOTE: Stability is determined based on the storage conditions in section D.3.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Homogenize the liver sample and blank tissue using a food processor.

b. Weigh 20 g ± 0.1 g of the homogenized liver into a 200 mL centrifuge bottle.

c. Perform a 0.1 ppm recovery by adding 1 mL of fortification standard solution to 20 g of blank tissue.

d. Add 10 mL of water and 500 mg trypsin.

e. Adjust to pH no less than 8 nor more than 8.5 by addition of 10% w/v sodium carbonate solution.

f. Incubate in 40° C water bath for 3 hours. (This is a convenient stopping point. Samples may be refrigerated and stored overnight or weekend.)

g. Cool to room temperature. (Warm, if refrigerated.)

h. Add 10 mL 10% w/v sodium carbonate solution and mix.

i. Add 100 mL ethyl acetate and macerate for 3 min with the tissuemizer.

j. Centrifuge for 2 min at 10° C, 2000 rpm.

k. Decant the ethyl acetate into a 500 mL separatory funnel via a vacuum suction apparatus. Clean the decanting tube between each sample with ethyl acetate.

l. Repeat steps i-k adding the second volume of ethyl acetate to the first extract.

m. Add 50 mL salt-saturated sodium carbonate solution (5% w/v) and shake vigorously for 1 min.

n. Discard the aqueous layer.

NOTE: If a precipitate forms in the aqueous layer, filter the layer plus precipitate into a beaker through a layer of glass wool contained in a funnel. Use ethyl acetate to wash precipitate and return the cleaned aqueous/ethyl acetate layers to the separatory funnel. The aqueous layer can then be drained off and discarded.

o. Add 50 mL 0.125M ammonium acetate buffer and shake for 1 min.

p. Transfer the ammonium acetate layer (lower layer) into a 250 mL separatory funnel.

q. Repeat steps o and p, adding the second ammonium acetate extract to the first extract.

r. Discard the ethyl acetate layer.

s. Add 10 mL ethyl acetate to the ammonium acetate buffer and wash gently for 10 sec.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

t. Transfer the ammonium acetate layer (lower layer) into a 250 mL round-bottom flask and evaporate all remaining ethyl acetate from the buffer solution. This takes approximately 20-30 min on the rotary evaporator. There must be no ethyl acetate odor remaining.

u. Transfer into a 100 mL volumetric flask and bring to volume with 0.125M ammonium acetate buffer solution.

v. Filter through a Whatman GF/F filter paper, discarding the first few mL of filtrate.

w. Elute 20 mL of filtrate through a pre-washed Sep-Pak C_{18} cartridge. Discard filtrate. (Refer to section C, Reagent and Solution List, item s.i, for prewash instructions.) At this point, sample cartridge may be refrigerated overnight or over a weekend before proceding to next step.

x. Wash the cartridge with 3 mL of water and discard the washing.

y. Elute halofuginone from the cartridge with 5 mL methanol into a 15 mL conical centrifuge tube.

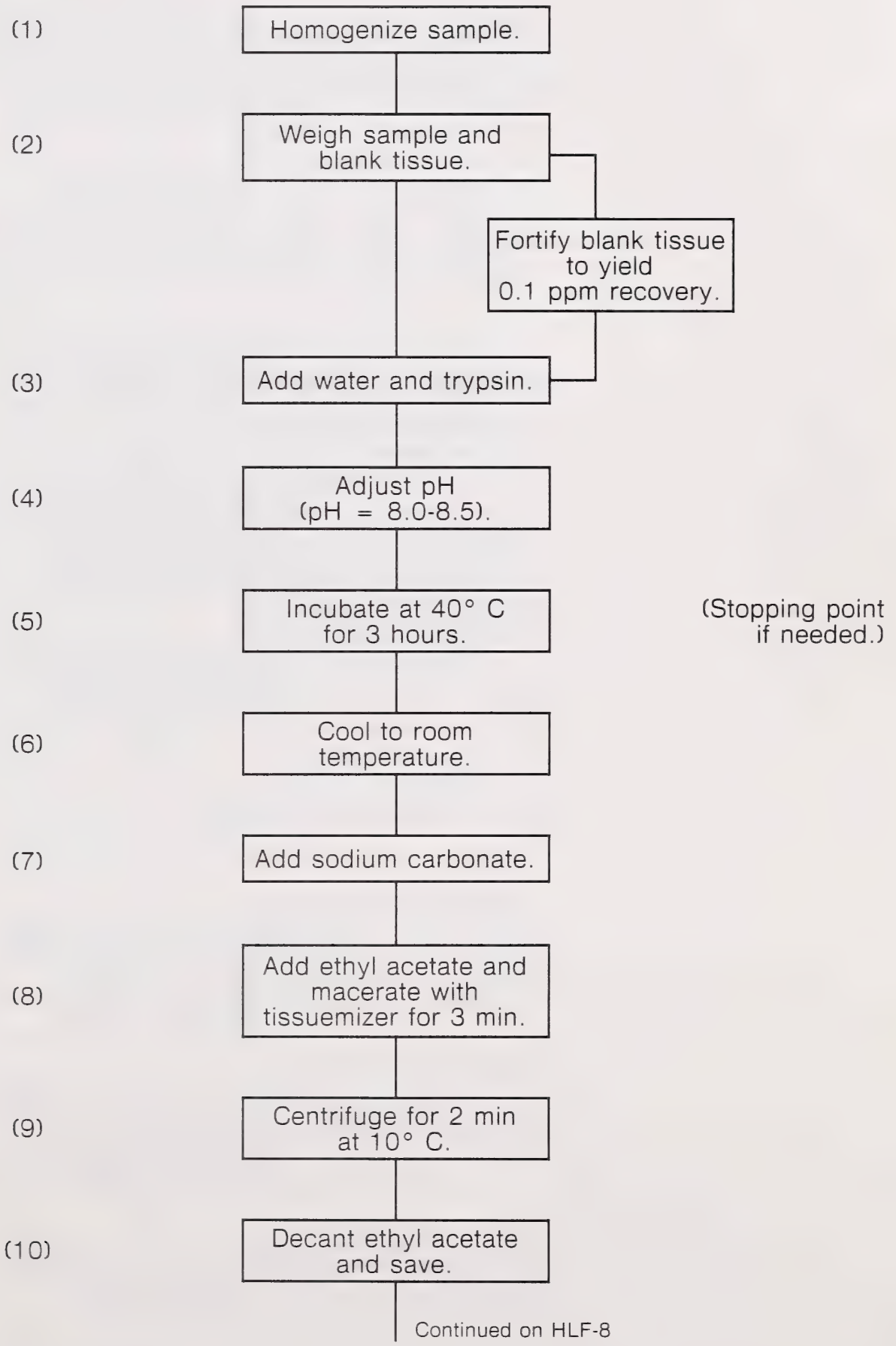
z. Under a gentle stream of nitrogen, evaporate to 1 or 2 mL. Pass through a Millex® HV 0.45 filter unit. Rinse the unit with a small amount of methanol and continue to evaporate sample to dryness.

aa. Bring the residue to 400 μL with mobile phase, vortex, and transfer to WISP micro vial for HPLC analysis.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary



Continued on HLF-8

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

(11) Repeat steps 7-10 (adding step 7 to step 10 before proceeding).

(12) Add 50 mL salt-saturated sodium carabonate solution.

(13) Shake vigorously for 1 min.

(14) Discard the aqueous layer (refer to note in section E if a precipitate forms).

(15) Add 50 mL 0.125 M ammonium acetate buffer.

(16) Shake for 1 min.

(17) Transfer the ammonium acetate layer (lower layer) into a 250 mL separatory funnel.

(18) Repeat steps 14-16 (adding to step 17 before proceeding).

(19) Discard the ethyl acetate layer.

Continued on HLF-9

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

(20) Wash the ammonium acetate buffer.

(21) Transfer the ammonium acetate layer (lower layer) into a 250 mL round-bottom flask.

(22) Evaporate all remaining ethyl acetate (Roto-evap); there must be no ethyl acetate left.

(23) Transfer to a 100 mL volumetric flask and dilute to final volume with 0.125M ammonium acetate buffer.

(24) Filter the solution (from step 23).

(25) Elute 20 mL of filtrate through a pre-washed Sep-Pak C_{18} cartridge. Discard the filtrate.

(26) Wash the cartridge with 3 mL water. Discard the washing.

(27) Elute halofuginone from cartridge above with 5 mL of methanol.

Continued on HLF-10

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

(28) Collect eluate to a centrifuge tube.

(29) Evaporate to 1 to 2 mL with nitrogen.

(30) Rinse the unit with small amount of methanol and continue to evaporate to dryness.

(31) Bring the residue to 400 μL with mobile phase. Sample is ready for HPLC analysis.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

NOTE: Equivalent instrumentation may be substituted.

a. Wavelength: 243 nm.

b. Column: μ-BONDAPAK C_{18}, 30 cm × mm id, 100 × 2.1 mm C-8 HP #79916 MD microbore.

c. Mobile phase: Acetonitrile/0.25M ammonium acetate buffer/water 5:3:12 adjusted to pH 4.3 with acetic acid.

d. Flow rate: 0.125 mL/min.

e. Oven temperature: 40° C.

f. Pressure: 2,300-2,800 PSI.

g. Injection volume: 25 μL.

h. Retention time: 5-7 min.

i. Run time: 10 min.

j. Sensitivity: 0.02 AUFS.

k. Chart speed: 0.25 cm/min.

NOTE: Parameters may be changed to optimize chromatography.

2. Interferences

No known interferences.

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

The integrator calculates the nanograms of halofuginone in the sample. The ppm is calculated by the following formula.

$$\frac{\text{initial vol}}{\text{wt of sample}} \times \frac{\text{final vol}}{\text{aliquot}} \times \frac{\mu\text{g detected}}{\text{inj vol}}$$

i.e.,

$$\text{ppm halofuginone} = \frac{100\ \text{mL}}{20\ \text{g}} \times \frac{400\ \mu\text{L}}{20\ \text{mL}} \times \frac{\mu\text{g detected}}{50\ \mu\text{L}} = 2 \times \mu\text{g detected}$$

2. **References**

a. American Hoechst Corporation—NADA 130-951.

b. Hazleton Biotechnologies Corporation—Analytical Method 32 (1982).

c. Hazleton Biotechnologies Corporation—Analytical Method 98 (1984).

d. Oneida Research Services, Inc., "Confirmation Assay for Halfuginone in Chicken Liver Using Negative Ion Chemical Ionization Mass Spectrometry/Mass Spectrometry".

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Halofuginone*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: HALOFUGINONE

Analyst: _____

Date: _____

Recovery of This Set: _____

Running Average of 10 Latest
Acceptable Recovery Values: _____

COV of Running Average: _____

Standard Curve Data

$y = mx + b$

b = _____

m = _____

r = _____

F = _____

SAMPLE IDENTIFICATION	CALCULATION CONTROL POINTS					SAMPLE DATA			COMMENTS
	Sample Wt gm	Initial vol mL	Aliquot mL	Final Vol μL	Inject vol μL	Peak Height cm	μg halofug	ppm halofug*	
1. 0 μg Standard					50.0	0.0			
2. 0.025 μg Standard (1/2x)									
3. 0.05 μg Standard (1x)									
4. 0.10 μg Standard (2x)									
5. 0.1 ppm Recovery	20.0	100.0	20.0	400.0					
6. R926-									
7.									
8.									
9.									
10.									
11.									
12.									
13.									
14.									
15.									
16.									
17.									
18.									
19.									

*Recovery corrected

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Element	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*
Halofuginone	> 0.05	60-115	≤ 20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Mobile phase pH.	4.3 ± 0.05.
b. Glassware.	Must be silanized.
c. Trypsin addition.	500 mg ± 50 mg.
d. pH	8.25 ± 0.25.
e. Maceration time.	3-4 min.
f. Time constraint.	Steps i-t of section E must be completed in < 1 hr.
g. Ethyl acetate removal and bath temperature.	37° C ± 1° C; no odor remaining.
h. Filtrate volume for elution.	20 mL ± 0.02 mL.
i. Elution volume of methanol.	5 mL ± 0.01 mL.
j. Evaporation temperature.	< 40° C.
k. Final volume.	400 µL ± 10 µL.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each, on 3 separate days.

(a) Blank.

(b) 12.5 ng in 50 µL injection.

(c) 25 ng in 50 µL injection.

(d) 50 ng in 50 µL injection.

ii. Phase II: Fortified Samples—4 levels, 3 replicates each over a minimum of three days.

(a) Blank.

(b) 0.05 ppm.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

(c) 0.10 ppm.

(d) 0.20 ppm.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples from FSIS Western Laboratory (samples submitted by supervisor if only one laboratory is performing this test).

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst, or 20% of official samples analyzed (whichever is smaller).

ii. Blind samples (requires ''dummy'' forms).

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All % recoveries.

(d) Running average, standard deviation, and CV for recoveries.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability	a. Matrix: Chicken liver. b. Sample receipt size: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests. c. Condition upon receipt: Frozen. d. Sample storage: i. Time: 6 months. ii. Condition: Frozen.
6. Sample Set	a. Tissue blank. b. Fortified blank tissue at 0.10 ppm. c. Samples.
7. Sensitivity	a. Lowest detectable level (LDL): 0.05 ppm. b. Lowest reliable quantitation (LRQ): 0.05 ppm. c. Minimum proficiency level (MPL): 0.05 ppm.

II. CONFIRMATORY METHOD

Procedure for MS/MS Confirmation of Halofuginone Violations

a. Take 60 mL of the remaining filtrate from Determinative Method, section E, step w, and pass through a prewashed Sep-Pak C_{18} cartridge. Discard the filtrate coming through the cartridge.

b. Wash the cartridge with 3 mL of water and discard the washing. Be sure all water is removed before proceding to next step.

c. Elute the halofuginone from the cartridge with 5 mL of methanol and collect the methanol in a 15 mL conical centrifuge tube.

d. Evaporate to dryness under a gentle stream of nitrogen at 40° C.

e. Add 240 μL of mobile phase to the centrifuge tube and vortex thoroughly to dissolve the haofuginone residue. Pass through a 0.45 micron filter. Sample is ready for HPLC analysis at this point.

f. Prepare standards such that the peak height of the sample is bracketed by a standard of lower height and a standard of higher height. Use a 200 μL injection.

g. Chromatograph and collect the fraction of eluant containing the halofuginone peak. Also chromatograph the 0.1 ppm recovery and collect the halofuginone fraction.

h. Remove the acetonitrile from the eluate by rotary evaporation at 40° C.

i. Pass the sample through a prewashed Sep-Pak C_{18} cartridge and discard washing.

j. Wash the cartridge with 5 mL of water and discard washing.

k. Elute halofuginone from the cartridge into a 15 mL test tube with 5 mL methanol. Collect the eluate and evaporate to dryness under a gentle stream of nitrogen.

l. Send the sample, the 0.1 ppm recovery, and a 1 μg standard to:

Dr. James Sphon HHF 423
Division of Contaminants Chemistry
200 C Street, FOB #8
Washington, DC 20204

IVERMECTIN

Contents

I. Screening Method

A. Introduction 1
B. Equipment 3
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 8
G. Calculations 12
H. Hazard Analysis 13
I. Worksheet 14
J. Quality Assurance Plan 17

II. Determinative Method

A. Introduction 19
B. Equipment 20
C. Reagents and Solutions 21
D. Standards 22
E. Extraction and Derivatization 23
Confirmatory Section 26
F. Analytical Quantitation 27
G. Calculations 34
H. Hazard Analysis 34
I. Worksheet 34
J. Quality Assurance Plan 37

I. SCREENING METHOD

A. INTRODUCTION

1. Theory and Structure

This assay procedure for ivermectin in animal tissue was developed at Merck, Sharpe and Dohme Research Laboratories, Rahway, NJ.

A rapid analytical procedure has been developed for the isolation and determination of ivermectin in liver and muscle tissue. The analyte is extracted from the target tissue with iso-octane and cleaned up by precipitation of extraneous matrix residue using reduced temperature. A fluorescent derivative is prepared and analyzed using liquid chromatography. Recoveries of ovine, bovine, and porcine liver and muscle tissues fortified within a range of 7.5 ppm to 60 ppm averaged 75%, with a 12.5% coefficient of variation.

Avermectins, which are isolated from the mycelia of streptomyces avermitilis, are a broad-spectrum family of compounds that are potent agents at very low dosage levels. Ivermectin itself is a mixture of two homologues, not less than 80% 22.23-dihydroavermectin B1a or H2B1a and not more than 20% 22.23-dihydroavermectin B1b or H2B1b. The basic structure of these compounds is shown on the following page.

Radiometabolism studies conducted at Merck Laboratories have demonstrated that the H2B1b is metabolized more rapidly than H2b1a. In addition, these studies concluded that H2B1a is not rapidly metabolized and hence is the major residue found at the proposed withdrawal times. Thus 22.23-dihydroavermectin B1a appears to qualify as the correct marker substance, with liver tissue the target tissue.

Tolan and co-workers (1980) developed an HPLC method using flueorescence detection for avermectin in plasma. Modification of the Tolan method to apply it to tissue resulted in a procedure that is shorter and more reproducible. The method determines H2B1a rapidly at a lower limit of reliable measurement of 10 ppb or less. The procedure involves conversion of H2B1a to the fluorescent derivative, which is readily chromatographed and sensitively and selectively detected.

NOTE: This analysis is intended as a rapid screening method and a trade for speed was made at the expense of sample clean-up. Liver samples in particular and muscle samples to a lesser degree produce a late eluting peak. The derivatization step produces early eluting peaks in both tissues. Both types of interference can affect quantitation. However, there are critical control points that can maximize analytical performance.

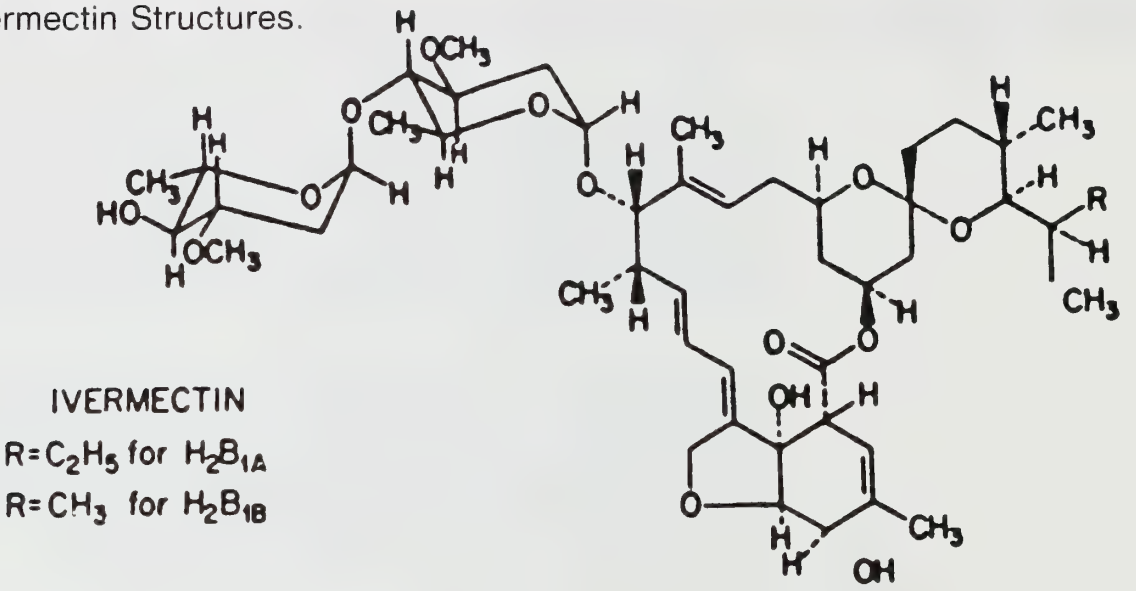
2. Applicability

The method developed has been applied to liver samples as well as kidney, muscle, fat, and plasma (Tway, et al., 1981). Tissue from cattle, sheep, horses, and swine have been successfully assayed.

I. SCREENING METHOD

A. INTRODUCTION (Continued)

Figure 1 Ivermectin Structures.



I. SCREENING METHOD

B. EQUIPMENT

1. Apparatus

a. Ultrasonic bath: Model B5200R1, Branson Ultrasonics Corp., Danbury, CT 06810-1961.

b. N-Evap: Model No. 112, Organomation Assoc Inc., Berlin, MA 01503.

c. High temperature oil bath: Model 160 High Temp Bath, Fisher Scientific, Fair Lawn, NJ 07410.

d. 50 mL polypropylene tubes: Part No. 222-3937-G80, Evergreen Scientific, Los Angeles, CA 90058.

e. 15 mL glass centrifuge tubes: Pyrex No. 8084, Corning Glassworks, Corning, NY 14831.

NOTE: The condition of the 15 mL glass centrifuge tubes affects analytical performance. Sylate all glass tubes before use. This makes them easier to clean and covers reactive sites that can absorb ivermectin.

f. Plug Tite caps: Catalog No. 127-0019-200, Elkay Laboratory, Shrewsbury, MA 01545.

g. Centrifuge: Sorvall T 6000B, DuPont Co., Newtown, CT 06470.

h. Solid-phase extraction cartridge: Waters Silica Gel, part no. 51900, Waters Chromatography Division, Milford, MA 01757.

i. Pipet aid: Catalog no. 4-00-110, Drummond Scientific Co., Broomall, PA 19008.

j. Laboratory blender and control: Catalog nos. 3392-C10 and 3392-C40, Thomas Scientific, Swedesboro, NJ 08065-0099.

k. Mechanical shaker and box carrier: Eberbach 610 Shaker and 6040 Box Carrier, catalog nos. 8287-C30 and 8287-J20, Thomas Scientific, Swedesboro, NJ 08065-0099.

l. Meat grinder: Hobart Model No. 4612, Hobart Corp., Troy, OH 45373.

2. Instrumentation

a. Liquid chromatograph: Waters M-600A pump, Model 710B Wisp Autosampler, Model CX 4 column heater, Model 420A fluorescence detector, Waters Associates, Inc., Milford, MA 01757.

b. Analytical column: DuPont Zorbax ODS 150 mm × 4.6 mm id, DuPont Co., Wilmington, DE 19898.

c. Pre-column: Brownlee Labs Spheri-5 RP-18, 30 mm × 4.6 mm 5pm, Applied Biosystems, Inc., Foster City, CA 94404.

I. SCREENING METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. 1-Methylimidazole, Aldrich Chemical, catalog no. M5, 083-4, Aldrich Chemical Co., Inc., Milwaukee, WI 53233.

b. Acetic anhydride, Mallinckrodt catalog no. 2420-500, Mallinckrodt, Inc., Paris, KY 40361.

c. N,N-Dimethyl formamide, Fisher Chemical catalog no. D119-500, Fisher Scientific, Fair Lawn, NJ 07410.

d. Derivatizing reagent: Mix 2 parts 1-methylimidazole, 3 parts acetic anhydride, and 9 parts N, N-dimethyl formamide in a clean, dry 15 mL glass centrifuge tube with a vortex mixer. Mix prior to use and discard unused portion.

NOTE: The derivatizing reagent is sensitive to alkaline and acidic residues, so a rigorous cleaning regimen for the glass tubes should be used. Remove all sample residue by hand—do not machine-wash tubes. Soak in methylene chloride for 2 hr; then soak in detergent solution for 2 hr. Rinse with hot tap water, distilled water, acetone, and methanol before drying.

e. Methanol, LC grade.

f. Acetonitrile, LC grade.

g. Iso-octane, LC grade.

h. Acetone, LC grade.

i. Chloroform, LC grade.

j. Water, distilled.

k. High temperature bath oil, Fisher Chemical catalog no. 02-20, Fisher Scientific, Fair Lawn, NJ 07410.

l. Cylon CT, Supelco catalog no. 3-3065, Supelco, Inc., Bellefonte, PA 16823-0048.

m. Mobile phase: 96% methanol, 3% distilled water v/v.

n. Acetone/water extraction solution: 50% acetone, 50% water v/v.

I. SCREENING METHOD

D. STANDARDS

1. Source	Ivermectin standard, catalog no. L$640,471-076P004. Merck, Sharpe and Dohme Rahway, NJ 07065
2. Preparation of Standards	a. Follow preparation instructions accompanying standards received from Merck, Sharpe and Dohme to obtain a stock solution of approximately 125 μg/mL. b. Make two intermediate dilutions to obtain a spiking solution of 0.5 μg/mL.
3. Storage Conditions	The stock solution and all dilutions of same should be stored in polypropylene tubes at $-20°$ C.
4. Shelf Life Stability	a. Spiking solution: 90 days. b. Stock (from Merck): 1 year.

I. SCREENING METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

a. Accurately weigh 2.5 g ± 0.1 g of the test sample and place in a 50 mL polypropylene tube.

b. For each sample, prepare, as described in a, a blank tissue fortified with 75 μL of 0.5 μg/mL standard solution to yield a recovery of 15 ppb.

c. Add 15 mL of acetone/water solution (refer to section C, Reagent and Solution List, item n).

d. Add 15 mL of iso-octane, cap the tube, and shake it by hand to disperse the tissue.

e. Using a mechanical shaker, shake the tube for 10 min.

f. Centrifuge for 10 min at 2000 rpm.

g. Transfer the iso-octane (top solvent layer) to a clean 15 mL glass centrifuge tube.

h. Reduce the iso-octane to about 0.5 mL with an N-Evap (60° C bath).

i. Reextract the sample-acetone/water solution two more times with 15 mL portions of iso-octane as previously described. Reduce the third and final 15 mL portion of iso-octane completely.

   NOTE: It is very important to transfer only the iso-octane layer during the tissue extraction step. To avoid the lower layer, transfer only 13-14 mL from each of the three 15 mL iso-octane passes. This is the most efffective way to manage late-eluting peaks.

j. Add 4 mL of preheated acetonitrile (60° C) to the waxy residue in the 15 mL glass centrifuge tube.

k. Stopper the tube and vortex-mix.

l. Return the tube to the N-Evap bath (60° C) for 5 min.

m. Vortex-mix the tube to dissolve matrix residue and sonicate for 2 min.

n. Place the tube in a freezer (0° C) for 1 hr.

o. Remove the tube from the freezer and centrifuge at 1500 rpm for 5 min.

p. Decant the acetonitrile into a clean 15 mL glass centrifuge tube and discard the sample residue. Completely reduce the acetonitrile using an N-Evap (60° C).

q. Prepare the external standard by pipetting an appropriate amount of ivermectin spiking standard (refer to section D.2.b) into a clean 15 mL glass centrifuge tube and completely reducing the methanol carrier, using an N-Evap (60° C).

I. SCREENING METHOD

E. EXTRACTION PROCEDURE (Continued)

r. Add 150 μL of derivatizing agent to each tube. Cap, vortex for 20 sec, and sonicate for 30 sec.

s. Centrifuge the tubes for 1 min at 1500 rpm.

t. Place the tubes, submerged 2-3 cm, into the high temperature oil bath (96° C) for 1.5 hr.

u. Remove the tubes from the oil bath and wipe off excess oil. Add 1 mL of chloroform to each tube.

v. Pre-wet the silica Sep-Paks with 3-4 mL of chloroform.

w. Decant the contents of the tube onto the Sep-Pak and rinse the tube with 3 mL of chloroform onto the Sep-Pak.

x. Pass 8 mL of chloroform through the Sep-Pak after the initial 3-4 mL has passed.

y. Retain all eluted chloroform in a clean 15 mL glass centrifuge tube. Completely reduce the chloroform and take to final volume with 1 mL of methanol.

z. Cap, vortex, and sonicate the tube.

aa. Place the tubes in a freezer (0° C) for 30 min.

bb. Remove cold tubes and centrifuge at 1500 rpm for 5 min. Encapsulate sample for liquid chromatographic determination.

I. SCREENING METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. Flow rate: 1.8 mL/min

b. Injection volume: 100 μL.

c. Column temperature: 30° C.

d. Excitation wavelength: 360 nm.

e. Emission wavelength: 425 nm.

f. Detector attenuation: 2.

g. Recorder speed: 5 mm/min.

h. Recorder range: 50 mv.

i. Run time: 20 min.

NOTE: The late eluting peak mentioned in section A.1 has a retention time of about 60 min. The run time for each injection is 20 min. This causes the late eluter to coelute with an injection front. These parameters can be changed if they do not work. Flush the system with 100% methanol for 40 min after use to remove all late eluters.

Late eluting peaks cannot be avoided, but they can be minimized by transferring the iso-octane properly during tissue extraction. Manipulating mobile phase and run time parameters can mask late-eluting peaks under injection fronts.

NOTE: Proceed to Part II, Determinative Method, for any positive samples found at or above 80% action level.

2. Interferences

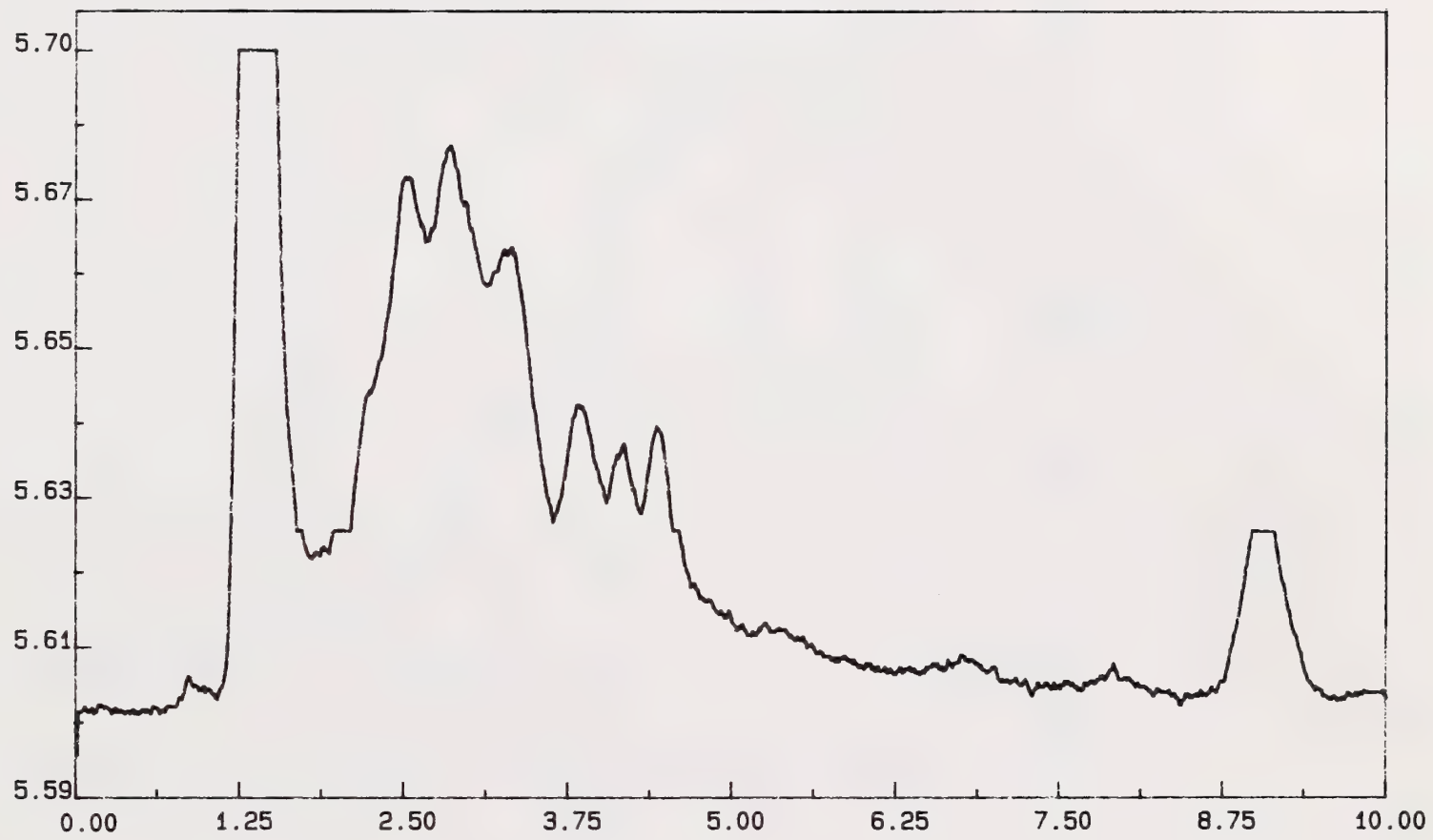
Avoid lipid material in the final extract.

I. SCREENING METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. Sample Chromatograms

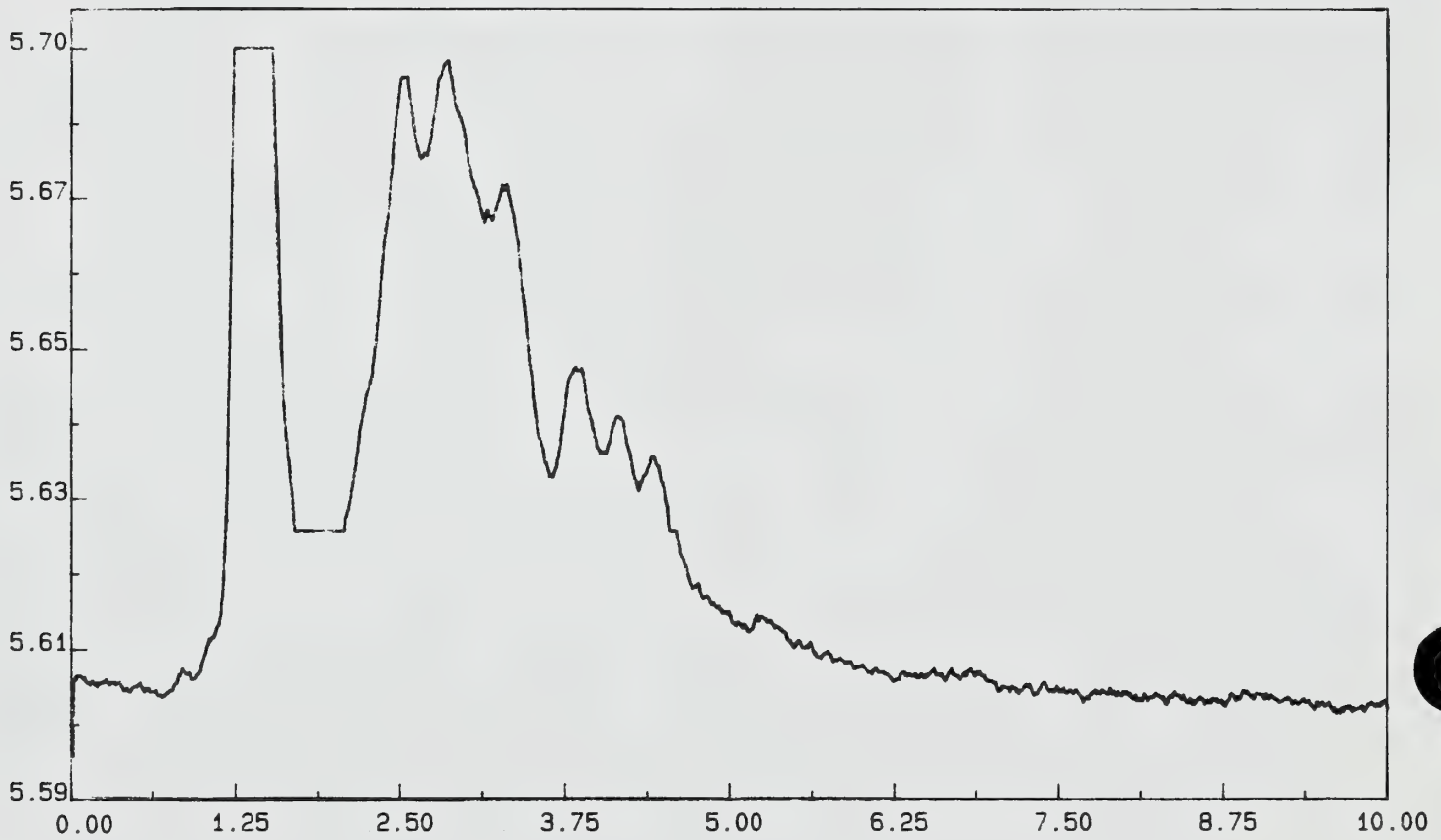
Ivermectin B1a Recovery (30 ppb).



I. SCREENING METHOD

F. ANALYTICAL QUANTITATION (Continued)

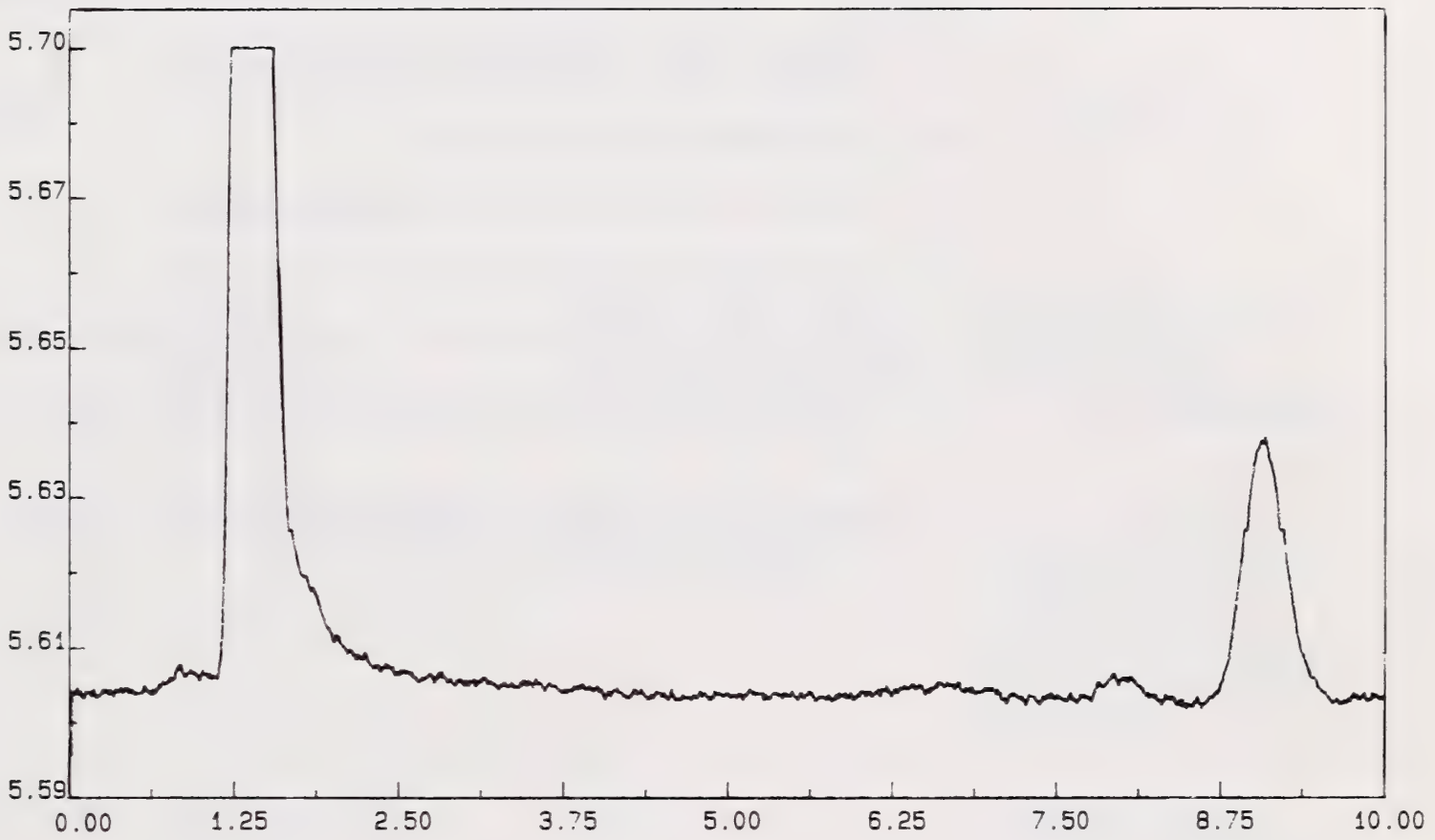
Ivermectin B1a Blank.



I. SCREENING METHOD

F. ANALYTICAL QUANTITATION

Ivermectin B1a Standard (30 ppb).



I. SCREENING METHOD

G. CALCULATIONS

1. Procedure

$$\frac{A}{B} \times D \div E = C$$

where:

A = concentration of standard representing ng/g.

B = peak height of standard in mm.

C = concentration of sample representing ng/g.

D = peak height of sample in mm.

E = running 10 average recoveries from the screening method.

2. References

a. Tway, P. C., Wood, J. S., and Downing, G. V., *J. Agric. Food Chem.*, 29, 1059-1063. 1981.

b. Method validation NADA 131-392; NADA 135-008. Ivermectin application: Merck & Co., Inc., Rahway, NJ 07065.

I. SCREENING METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination and Confirmation of Ivermectin Residue in Animal Tissue		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Extraction solvents Acetone Acetonitrile Iso-octane Chloroform Hexane	Solvents are corrosive and flammable. Vapors can cause severe skin, eye, and respiratory irritation.	Work should be in an efficient fume hood away from heat-generating devices.
	Standard solutions Ivermectin homologues	Weak teratogen and possible mutagen.	These biological compounds should be handled with the utmost care since very little toxicological data concerning them is available.
4. Disposal Procedures	Organic solvent mixtures	See above	Reduce volume by partial evaporation in a fume hood. Segregate chlorinated from non-chlorinated solvents before disposal by the contractor or in-house specialist.

I. SCREENING METHOD

I. WORKSHEET

The worksheet on the facing page, *Ivermectin Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

IVERMECTIN ANALYSIS—SCREENING

Analyst: _____

Date: _____

CONDITIONS

15 cm length × 4.6 mm id ZORBAX CDS C_{18} column. Mobile phase 3%. H_2O in CH_3OH (v/v).
Column temperature 30° C.
Flow 1.8 mL/min; 50 mL inj.; 15 ppb STD.

STD Conc. 500 μg/mL.
Matrix spike 15 ppb.

Percent recovery _____

	Sample ID	Critical Control Points									
	Procedure Key	Sample Weight 2.5 (g)	Spiking Volume 75 μL	Shaking Time 10 min	Derivative Reagent 0.15 μL (150 μL)	Reaction Time 1.5 hr	Reaction Temperature 96° C	Final Volume 1 mL	Peak Height	ppb Found on % Recovery	Comments
	External Std										
	Fortified Control										
	Sample #( ) at 15 ppb										
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											

I. SCREENING METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppb)*	*Acceptable Recovery (%)*	*Repeatability CV (%)*
Ivermectin	> 7.5	60-110	< 20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	2.5 g ± 0.1 g.
b. Upper layer transfer	No lower layer transferred.
c. Solids removal	Remove all solids.
d. Placement of tubes in oil bath.	2-3 cm below surface.
e. Chloroform evaporation	Residue completely dry.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Acceptable standard curves on 3 successive days using standards at 0, 7.5, 15, 30, and 60 ppb.

ii. Phase II: Self-fortified samples—Four recoveries from each of bovine, swine, and ovine liver on three sets 0, ½x, x, and 2x, where x is equal to the action level for each species in ppb.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Check samples for analyst accreditation.

(a) Ten liver samples fortified between 0 and 60 ppb, submitted by supervisor or LSO.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample monthly per analyst.

I. SCREENING METHOD

J. QUALITY ASSURANCE PLAN (Continued)

ii. Blind check samples or random duplicates chosen by the supervisor or LSO. Records to be maintained by the analyst and reviewed by the supervisor and LSO:

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

(d) Running average, standard deviation, and CV for all recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability	a. Matrices: Liver, Muscle, Kidney (swine, bovine, equine, ovine, chevon). b. Sample receipt size: 12 oz minimum. c. Condition upon receipt: Frozen. d. Sample storage: i. Time: 6 months. ii. Condition: Frozen.
6. Sample Set	a. Recovery. b. Thirteen samples and a standard.
7. Sensitivity	a. Lowest detectable level (LDL): 2 ppb. b. Lowest reliable quantitation (LRQ): 7.5 ppb. c. Minimum proficiency level (MPL): 5 ppb.

II. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

The subject of this method is the confirmation of residues determined by the method in Part I. Parts of the clean-up tissue are converted to (1) the monosaccharide and (2) the aglycone, with parts of the original remaining. All three portions are next converted to fluorescent derivatives and the liquid chromatography is run as in the previous procedure. In the new procedure the isolation of the derivative is by solvent extraction instead of Sep-Pak treatment and hence interferences would have to both Sep-Pak and solvent extract like ivermectin. The new compounds are formed by treatment with 1% sulfuric acid in isopropanol and methanol respectively. Thus a potential interference would have to form two new compounds with exactly the same chromatographic properties as those of ivermectin in two very different mediums. All three peaks may be semi-quantitated.

The tissue is homogenized with acetone/water and the dihydro B1a is extracted with iso-octane with the tissue present. Following removal of the iso-octane, solvent-solvent distributions into acetonitrile out of hexane and into hexane out of acetonitrile-water are performed. Solvent is removed and fluorescence produced by heating at 95° C in imidazole reagent. After adding chloroform, the reaction mixture is washed through a silica gel Sep-Pak, the solvent removed, and LC analysis on Zorbax C-18 with fluorescent detection is performed.

2. Applicability

Refer to Screening Method, section A.

II. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Balance: analytical, capable of weighing 1 mg accurately.

b. Balance: capable of weighing 5 g accurately.

c. Bath, water: variable temperature, 40°-80° C.

d. Bath, oil: 95°-100° C.

e. Centrifuge: IEC Model HN-S-II, or equivalent, with six-place rotor, for 15 mL and 50 mL tubes.

f. Centrifuge tubes: 15 mL and 50 mL glass with polypropylene stoppers.

g. Centrifuge tubes: 50 mL polypropylene, Corning #25331 (used for storing standards only), or equivalent.

h. Centrifuge tubes: 15 mL sylated (refer to Determinative Method, section B.1.e, NOTE).

i. Dispensing pipetters: 10, 15, and 20 mL.

j. Gloves: disposable PVC, Fisher Scientific, or equivalent.

k. Freezer: capable of −20° C.

l. Graduate cylinders: 25 mL and 500 mL.

m. Pipets: disposable.

n. Pipets, graduated: 1.0 mL and 2.0 mL.

o. Parafilm.

p. Pipets, volumetric: 0.5, 1, 2, 3, 4, and 5 mL.

q. Reciprocating shaker: Eberbach, J. T. Baker, catalog no. 8287-E30, or equivalent.

r. Sep-Pak: Waters Associates.

s. Sorvall Omnimixer, Model 17105, or equivalent.

t. Syringes: 50 μL, 250 μL, and 5 mL.

u. Tape: ½", Ace Scientific, #13-2956.

v. Ultrasonic bath: Sonogen Automatic Cleaner, Bronson Model 520, or equivalent.

2. Instrumentation

Refer to Screening Method, section B.2.

II. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Acetic anhydride: Baker, reagent grade, or equivalent.

b. Acetonitrile: Fisher or Mallinckrodt, nanograde, or equivalent.

c. Dimethyl formamide (DMF): Baker.

d. Hexane: Burdick and Jackson, distilled in glass, or Mallinckrodt, nanograde, or equivalent.

e. Isobutyl alcohol: Burdick and Jackson, distilled in glass, or equivalent.

f. Isopropyl alcohol: Burdick and Jackson, distilled in glass, or equivalent.

g. Methanol: Burdick and Jackson, distilled in glass, or Mallinckrodt, nanograde, or equivalent.

h. Methylene chloride: any grade available.

i. Methylene chloride: Burdick and Jackson, distilled in glass, or equivalent.

j. 1-Methylimidazole: 99%, Aldrich Chemical Co., or equivalent.

k. Nitrogen: Matheson, extra-dry compressed, or equivalent.

l. Sylon CT: Supelco, Inc.

m. 2, 2, 4-Trimethylpentane (iso-octane): Mallinckrodt, nanograde, or equivalent.

n. Water: double-distilled, deionized.

o. Derivatizing reagent: Mix 0.2 mL of 1-methylimidazole with 0.3 mL acetic anhydride and 0.9 mL DMF. Make just before use.

p. Acetone/water: 50% v/v.

q. Methanol/water: Dilute 100 mL of distilled/deionized water to 2 L with methanol. Deaerate by slowly bubbling nitrogen through for 10 min.

r. Methylene chloride/hexane/isobutyl alcohol: Fill a 500 mL stoppered graduate to the 200 mL mark with methylene chloride. Add hexane to the 500 mL mark and add 20 mL isobutyl alcohol.

s. 1% isobutyl alcohol in hexane: Dilute 5 mL of isobutyl alcohol to 500 mL in graduated cylinder.

t. 1% sulfuric acid in isopropanol: Pipet 0.5 mL of sulfuric acid carefully into about 40 mL of isopropanol in a 50 mL volumetric flask. Dilute to mark with isopropanol. Prepare fresh before use.

u. 1% sulfuric acid in methanol: Pipet 0.5 mL sulfuric acid carefully into 40 mL of methanol in a 50 mL volumetric flask. Dilute to mark with methanol. Prepare fresh before use.

II. DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standard Solution	Dilute a standard ivermectin solution in methanol until each 2 mL of the solution contains 5 times the number of nanograms of ivermectin as the level in the meat in ppb.

II. DETERMINATIVE METHOD

E. EXTRACTION AND DERIVATIZATION

1. Sample Preparation

a. Homogenize sample in a blender.

b. For each sample found positive in the screening method, weigh 3 separate portions into 50 mL screw-cap polypropylene centrifuge tubes.

i. Sample I: Weigh accurately 2.5 g ± 0.1 g of sample, which will be used in the quantitation of the final result.

ii. Sample M: Weigh accurately 5.0 g ± 0.1 g of sample, which will be used in the derivatization of the monosaccharide.

iii. Sample A: Weigh accurately 5.0 g ± 0.1 g of sample, which will be used in the derivatization of the aglycone.

2. Fortification Standard

a. Standard I: To 2.5 g ± 0.1 g of blank tissue, add 75 μL of 0.5 μg/mL standard solution to yield a 15 ppb recovery.

b. Standards A and M: To 5.0 g ± 0.1 g of blank tissue, add 150 μL of 0.5 μg/mL standard solution.

3. Sample Extraction

Applicable to liver and muscle tissues.

a. Add 15 mL of 50/50 acetone/water and 15 mL iso-octane to the 50 mL centrifuge tube.

b. Stopper the tube, shake well in a mechanical shaker for 10 min, and centrifuge for 10 min at 2000-2500 rpm.

c. Transfer the upper iso-octane layer to a second 50 mL centrifuge tube with a disposable pipet. Completely avoid the lower layer.

d. Break up the plug by using a vortex mixer and/or shaking.

e. Add 15 mL of fresh iso-octane and repeat the extraction, combining the iso-octane layers.

f. Blow the combined iso-octane layers down to a small volume using 80° C water bath and nitrogen purge.

g. Repeat the extractions with two more passes of 15 mL iso-octane, adding it in each case to the same tube that had the previous two iso-octane layers.

h. Again blow down as far as possible, using 80° C bath and nitrogen purge.

i. Add 6 mL of methanol to all samples and dissolve or resuspend completely with an ultrasonic bath and/or vortex mixer.

j. Place all samples in a refrigerator until thoroughly cooled. (At this point the sample is best left overnight in the refrigerator.)

II. DETERMINATIVE METHOD

E. EXTRACTION AND DERIVATIZATION (Continued)

k. Centrifuge the cold 50 mL tube for 5 min and decant off the clear supernatant to a fresh 15 mL centrifuge tube.

l. Wash the residue in the 50 mL tube from step k with 1 mL of methanol using a vortex mixer, centrifuge, and decant off the supernatant into the same 15 mL tube used in step k. Centrifuge the 15 mL tube and if necessary decant off into a new 15 mL tube to get a clear solution with which to continue the assay.

m. For muscle only, do a further 2 mL hexane extraction of the sample and add to the combined methanol.

n. Using a 60° C bath and nitrogen purge, blow off all the methanol.

o. Add 3 mL of hexane to the tube previously containing the methanol and use ultrasound to remove all material from the walls of the tube.

p. Add 4 mL of acetonitrile and repeat the ultrasound treatment.

q. Shake thoroughly, centrifuge 5 min, and move the lower acetonitrile layer to a fresh 15 mL tube.

r. Re-extract the hexane layer with a second 4 mL of acetonitrile and combine acetonitrile layers.

s. Extract the combined acetonitrile layers with 1 mL of hexane, centrifuge to clear, and move the upper hexane layer to waste by disposable pipet.

t. Blow the acetonitrile solution to < 1 mL using a 60° C water bath and nitrogen purge; then make up to 1 mL with fresh acetonitrile.

u. If necessary, use ultrasound to get a homogeneous mix.

v. Add 4 mL of distilled water (2 mL for kidney samples) and 5 mL of hexane and shake ca. 1 min. Centrifuge for 5 min.

w. Move the upper hexane layer to a fresh 15 mL tube using a disposable pipet. Avoid lower layer.

x. Repeat with 5 mL and then 4 mL hexane extractions, combining all three hexane extractions.

y. Blow to dryness (or as near dryness as possible) using a 40° C water bath and nitrogen purge. (To get completely dry, more heat may be necessary at the end of the evaporation. The bath may go as high as 80° C at this point.)

z. Take the residue up in exactly 1 mL of methanol, using vortex or ultrasound to dissolve.

NOTE: For Sample I and standard, proceed to steps bb through pp. For Samples A and M and standard, go to section E.4.

II. DETERMINATIVE METHOD

E. EXTRACTION AND DERIVATIZATION (Continued)

aa. Mix thoroughly and centrifuge for 5 min.

bb. Carefully blow off the methanol using a 60° C bath and nitrogen purge. Avoid spattering.

cc. Add 0.1 mL of freshly made acetic anhydride/methyl imidazole/DMF reagent to each sample tube and the A & M standard tubes using a 1.0 mL measuring pipet.

dd. Stopper, vortex, and centrifuge for a few seconds.

ee. Tape the stoppers in place and put all samples and standards in a well-stirred 95° C oil bath for 1 hr. The bottom of the tubes should be about 1″ below the oil surface.

ff. Remove the tubes, wash the oil off with acetone, and cool.

gg. Add about 1 mL of chloroform to each tube and vortex to mix.

hh. Wash a Sep-Pak cartridge with 3-4 mL of chloroform, using a syringe to force the liquid through the cartridge.

ii. Add the sample to the syringe by disposable pipet and force it through the cartridge.

jj. Wash the cartridge tube three times with chloroform, using 1 mL each time, adding the washes to the syringe and through the cartridge.

kk. Elute the column with a further 8-9 mL of chloroform (3, 3, 2 or 3 mL).

ll. Collect *all* the chloroform in a 15 mL centrifuge tube.

mm. Evaporate the chloroform off with a 60° C water bath and nitrogen purge. Get the residue completely dry.

nn. Pipet 0.5 mL of methanol (or other suitable quantity) into the tube and use a vortex mixer and ultrasound to completely dissolve the residue.

oo. Centrifuge briefly and inject 50 mL of the supernatant of each sample and standard into the LC.

NOTE: Suitable stopping places. *In a refrigerator at 4° C*—Only in methanol after step j. *In a freezer at −20° C*—After step ff, the derivatization, or after step nn, dissolution from chloroform. If stopping places other than the above are needed, assay stability at these stopping places should first be demonstrated.

II. DETERMINATIVE METHOD

E. EXTRACTION AND DERIVATIZATION (Continued)

CONFIRMATORY SECTION

4. Derivatization

a. Pipet exactly 0.5 mL of the solution from step 3.z into each of two fresh 15 mL tubes and blow completely dry with nitrogen in a 70° C bath. The dried samples generally look like a small drop of oil. All solvent must be removed.

b. Add 0.1 mL of 1% sulfuric acid in methanol to one of the samples [the A (aglycone) sample] and 0.1 mL of 1% sulfuric acid in isopropanol to the other [the M (monosaccharide) sample].

c. Vortex the sample for 10 sec, ultrasound it, and vortex again.

d. Let stand at room temperature for 16-18 hr (overnight).

e. To all A samples and A standards, add methanol to the 1 mL mark and quickly mix.

f. Add mixed solvent of 40% methylene chloride/hexane/isobutyl alcohol to the 7 mL mark.

g. Add distilled water to the 11 mL mark, shake for 1 min, and centrifuge for 5 min.

h. Move the mixed solvent upper layer to a fresh sylated 15 mL tube using a disposable pipet. Move as much of the upper layer as possible but absolutely no lower phase should be moved.

i. Repeat the extraction a second time with 6 mL of the mixed solvent. Combine extracts in the sylated tube.

j. Do the same extractions with the M samples, except that to avoid emulsions, the shaking must be only moderate and centrifugation should be 10 min.

k. Blow the M and A extracts to dryness. Again, all solvent must be removed and no moisture picked up. A bath temperature of 40° C is suitable for the M and A extracts.

l. Add 0.1 mL of the derivatizing reagent to M and A dry samples and standards. Vortex, tape the stoppers in place, centrifuge briefly, and place in a 95°-100° C oil bath for 1 hr, all at the same time.

m. Cool to room temperature. Add methanol to the 1 mL mark.

n. Add 1% isobutyl alcohol in hexane to the 7 mL mark and mix.

o. Add water to the 11 mL mark, shake 1 min, and centrifuge 5 min.

p. Move the upper layer as completely as possible to a fresh 15 mL tube. Move *no* lower layer.

II. DETERMINATIVE METHOD

E. EXTRACTION AND DERIVATIZATION (Continued)

r. Repeat the extractions with a second 6 mL of solvent. Combine extracts.

s. Blow completely to dryness with nitrogen purge in a 40° C water bath. At the end of this step, the temperature can be allowed to rise to 60°-70° C.

t. Take up residue in exactly 1 mL of methanol, using vortex mixing and ultrasound to dissolve. In all steps above, the M samples should be handled first and placed on the LC before handling the A samples. After extraction, protect the M samples from light as much as possible. If A samples are to be delayed significantly, they should be stored in a freezer at – 20° C before adding the methanol.

u. Centrifuge and inject 50 μL of the clear phase (M samples) onto the LC with one standard of each type, the unknowns, and finally the second standard, in that order.

v. Do the same with the A samples and standards.

5. Confirmation Criteria

To confirm the presence of ivermectin, the following criteria have to be met:

a. Both monosaccharide and aglycone peaks have to be present.

b. Retention time of both peaks should not differ from those in the fortified sample by more than ± 5%.

c. Quantitation of both monosaccharide and algycone should yield a recovery of ≥ 60%.

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

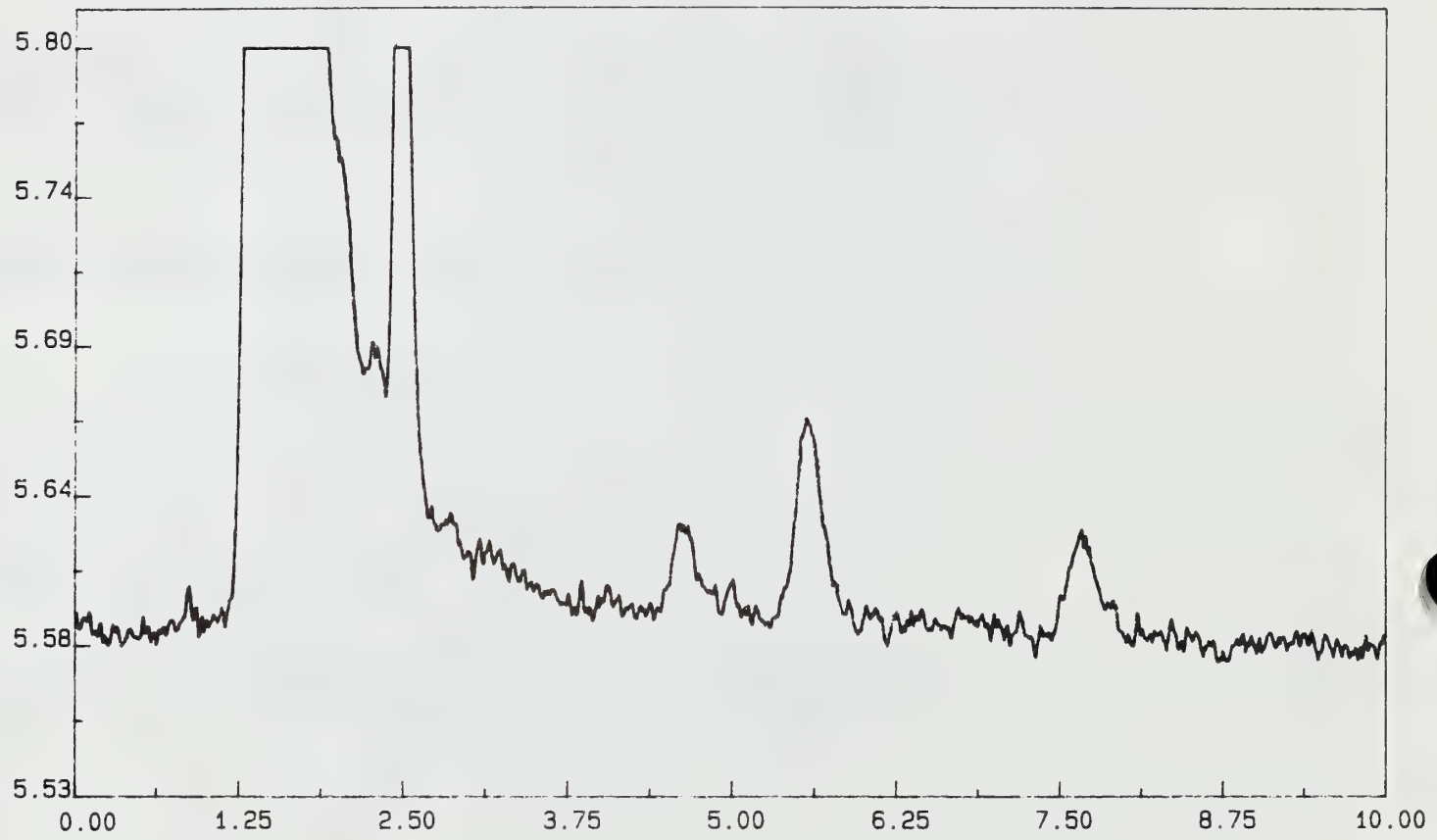
Refer to Screening Method, section F.

II. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

2. Sample Chromatograms

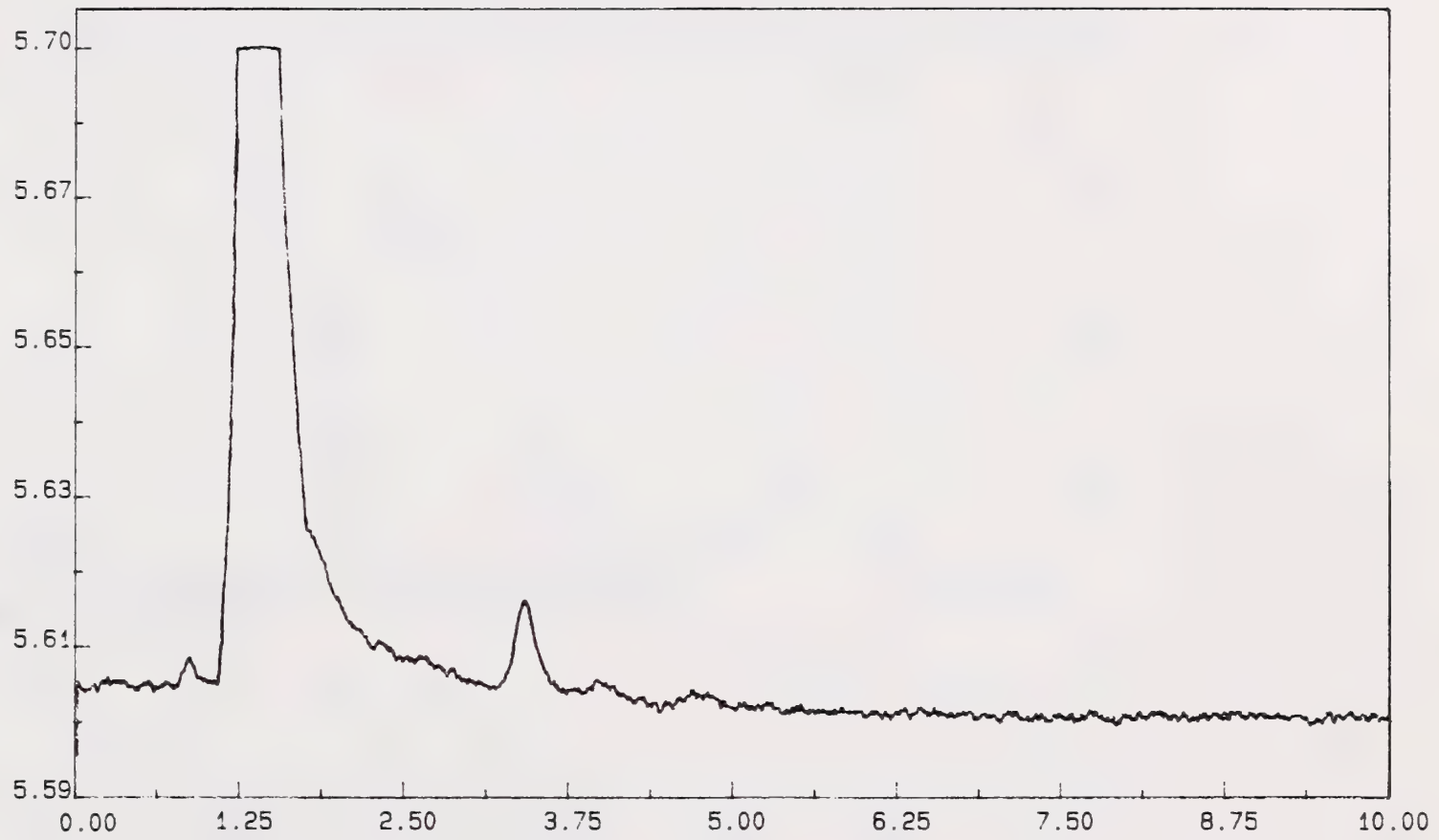
Ivermectin B1a Aglycone Recovery 15 ppb.



II. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

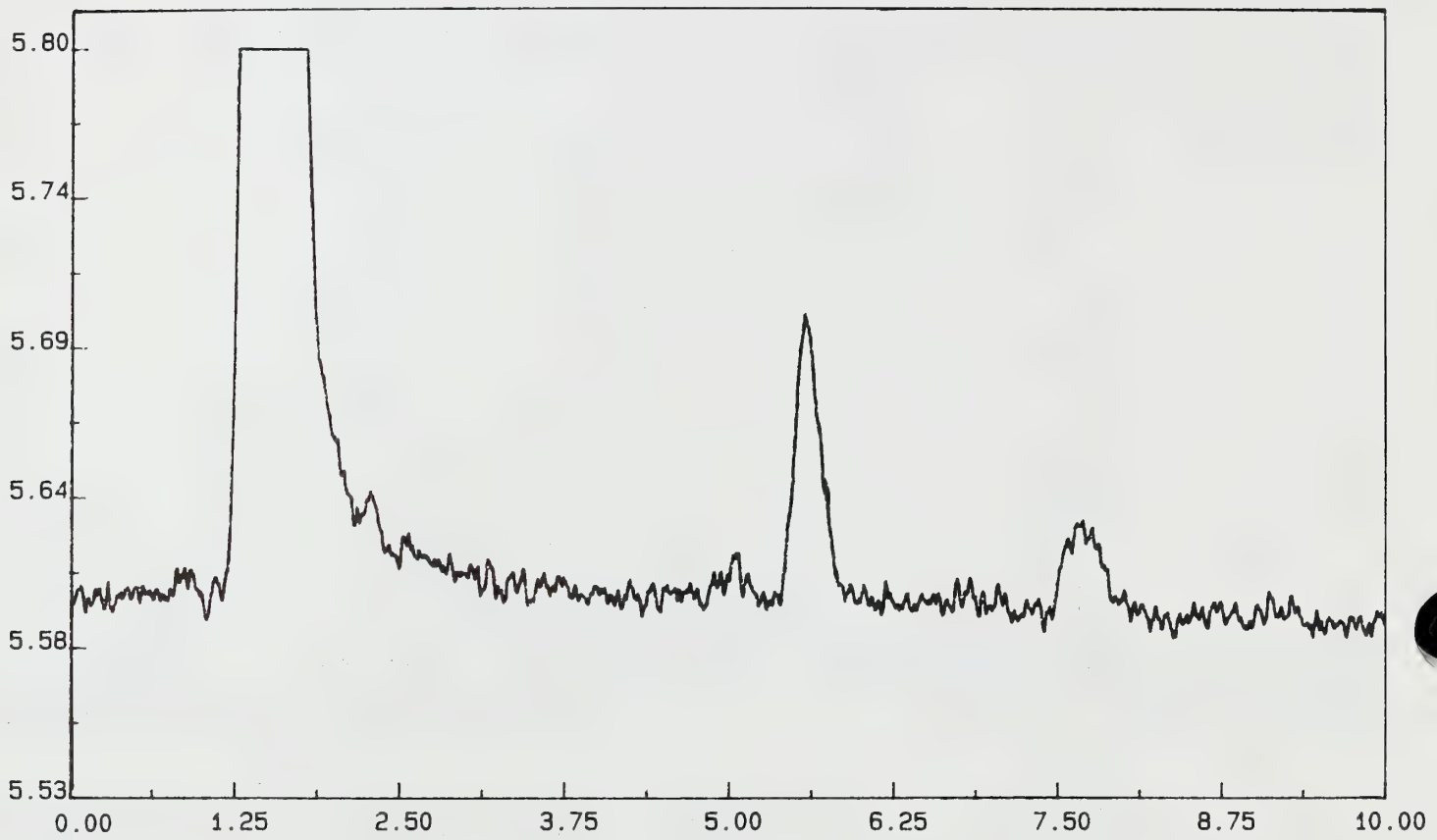
Ivermectin B1a Aglycone Blank.



II. CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION (Continued)

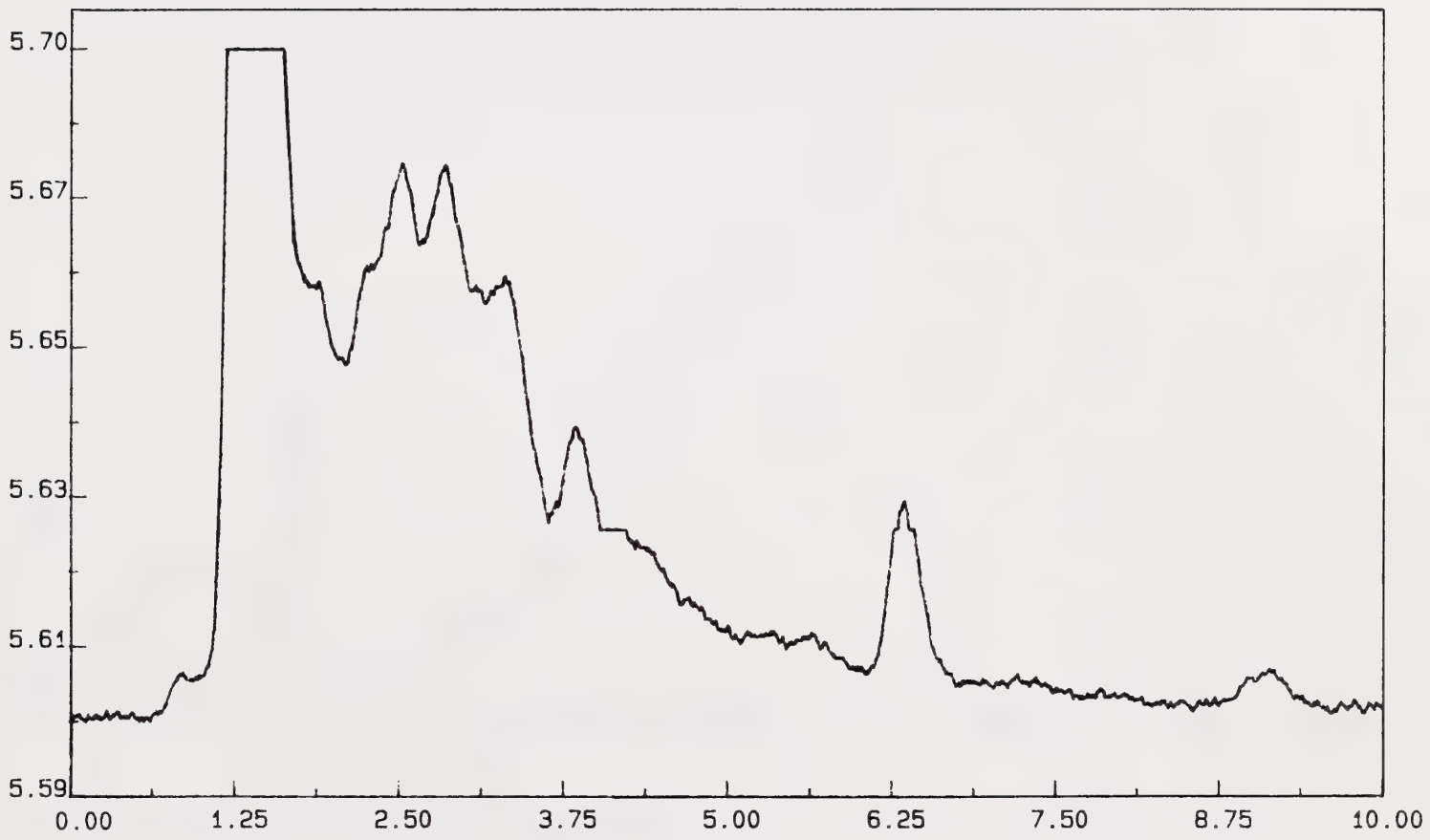
Ivermectin B1a Aglycone Standard 15 ppb.



II. CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION (Continued)

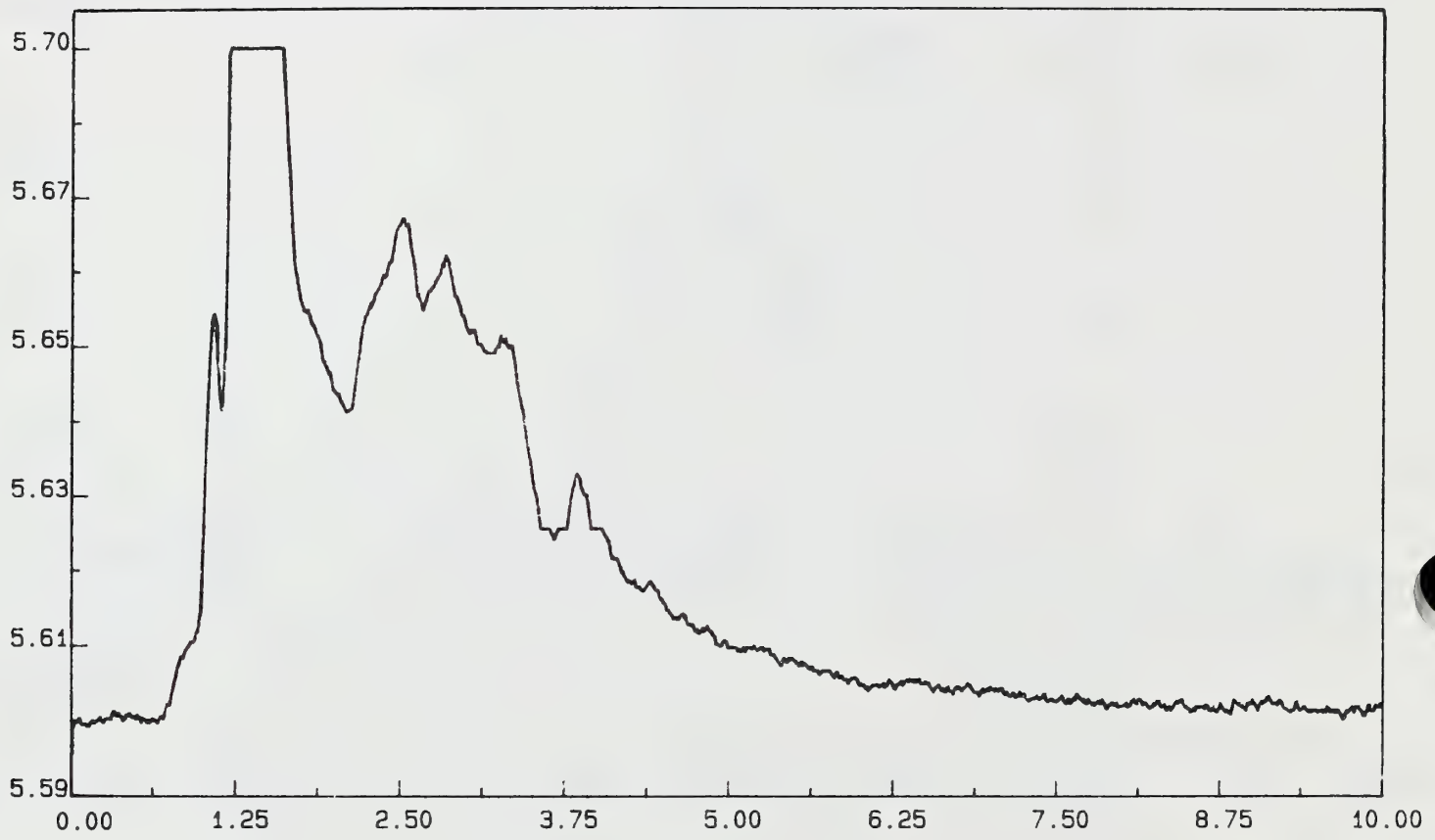
Ivermectin B1a Monosaccharide Recovery 30 ppb.



II. CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION (Continued)

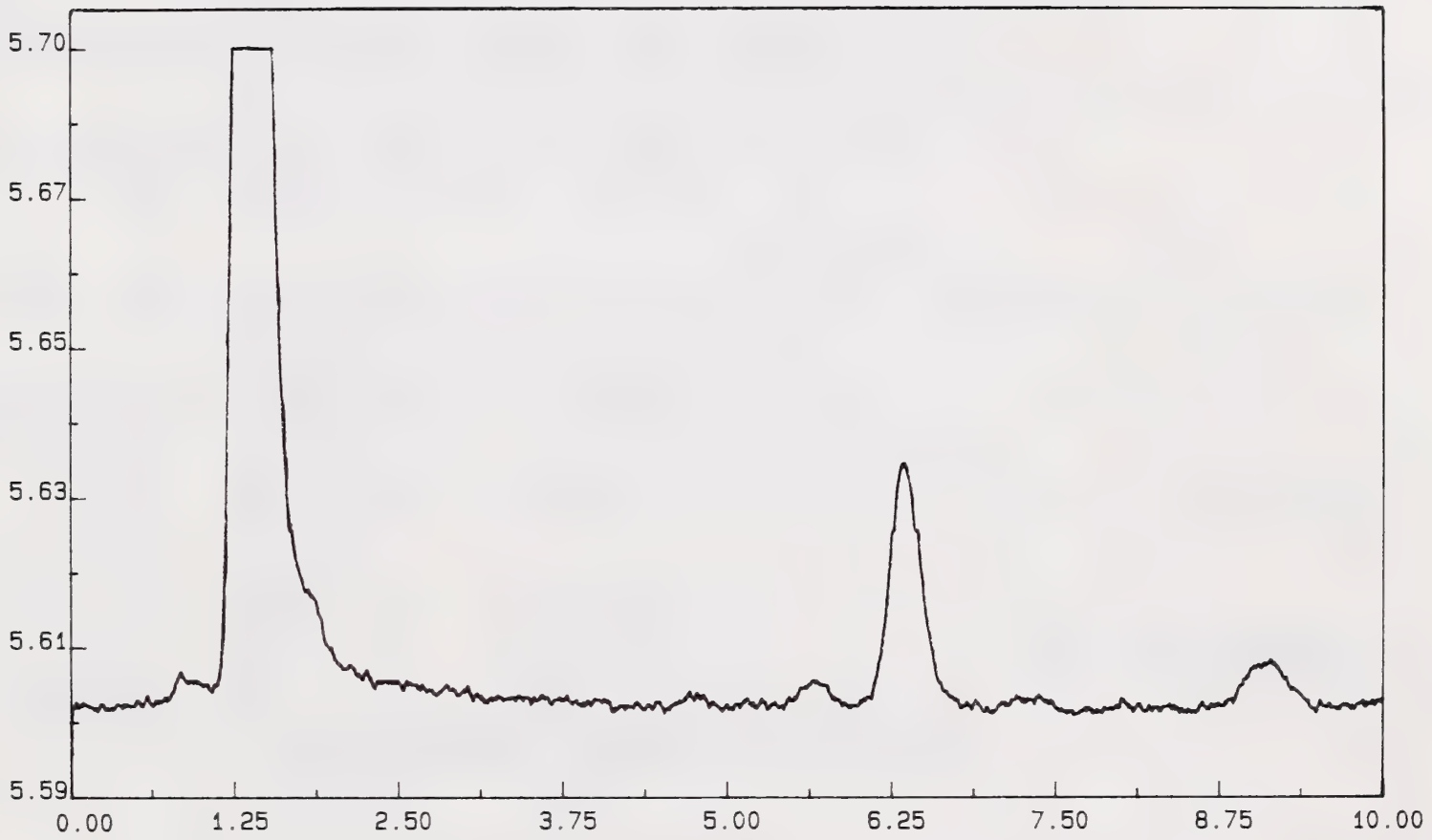
Ivermectin B1a Monosaccharide Blank.



II. CONFIRMATORY METHOD

F. ANALYTICAL QUANTITATION (Continued)

Ivermectin B1a Monosaccharide Standard 30 ppb.



II. CONFIRMATORY METHOD

G. CALCULATIONS

Procedure

a. Confirmation.

i. Examine the unknowns for the presence of the A, M, and I peaks at the same elution time as corresponding standard peaks.

ii. Average the two standard values for each sample and calculate each unknown as a percent of that standard.

iii. A value of 60% or more of both A and M is proof that the particular level of ivermectin is present in the original sample.

b. Quantitation.

i. Measure the peak height at the retention time of the derivative of dihydro-B1a as indicated by the standard.

ii. Refer to Screening Method, section G, for calculation procedures.

H. HAZARD ANALYSIS

Refer to Screening Method, section H.

I. WORKSHEET

The worksheet on the facing page, *Ivermectin Analysis,* can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

IVERMECTIN ANALYSIS—CONFIRMATION

Analyst: _____

Date: _____

CONDITIONS

15 cm length × 4.6 mm id ZORBAX CDS C_{18} column. Mobile phase 3%. H_2O in CH_3OH (v/v).
Column temperature 30° C.
Flow 1.8 mL/min; 50 mL inj.; 15 ppb STD.

STD Conc. 500 μg/mL.
Matrix spike 15 ppb.

Percent recovery _____

	Sample ID	Critical Control Points									
	Procedure Key	Sample Weight 2.5 (g)	Spiking Volume 75 μL	Shaking Time 10 min	Derivative Reagent 0.15 μL (150 μL)	Reaction Time 1.5 hr	Reaction Temperature 96° C	Final Volume 1 mL	Peak Height	ppb Found on % Recovery	Comments
	External Std										
	Fortified Control										
	Sample #( ) at 15 ppb										
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											

II. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Refer to Screening Method, section J.1. Applies only to the determinative portion. Refer to section E.5 for the confirmation criteria.

2. Critical Control Points and Specifications

Refer to Screening Method, section J.2, except for sample weight of samples A & M (5.0 g ± 0.1 g).

3. Readiness To Perform

a. Determinative (for the quantitation of sample I).

i. Familiarization.

(a) Phase I: Standards—Acceptable standard curves on 3 successive days using standards at 0, 7.5, 15, 30, and 60 ppb.

(b) Phase II: Self-fortified samples—Three duplicate recoveries from bovine liver on three sets of 0, 7.5, 30, and 60 ppb.

NOTE: Phase I and Phase II may be performed concurrently.

(c) Phase III—Check samples for analyst accreditation.

(1) Two sets of 7 bovine liver samples fortified between 7.5 and 60 ppb, submitted by supervisor or LSO.

(2) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

ii. Acceptability criteria.

Refer to Screening Method, section J.1.

b. Confirmatory (for the confirmation of sample A and sample M).

i. Familiarization.

(a) Phase I: Standards—Acceptable external standard curve on one day for each derivative at 0, 7.5, 15, 30, and 60 ppb.

(b) Phase II: Self-fortified standard—One set of recoveries at level as specified in Phase I for each matrix of interest.

NOTE: Phase I and Phase II may be performed concurrently.

(c) Phase III—Check samples for analyst accreditation. Refer to section 3.a.i.(c).

ii. Acceptability criteria.

Refer to confirmation criteria, section E.5.

II. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples	Refer to Screenig Method, section J.4.
5. Sample Acceptability and Stability	Refer to Screening Method, section J.5.
6. Sample Set	Refer to Screening Method, section J.6.
7. Sensitivity	Refer to Screening Method, section J.7.

LASALOCID IN BOVINE TISSUE

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 7
G. Calculations 9
H. Hazard Analysis 10
I. [Reserved]
J. Quality Assurance Plan 11

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Ten-gram aliquots of ground liver are extracted with 40 mL acetonitrile. A 23.5 mL portion of the extract is washed with hexane and the acetonitrile layer is evaporated to dryness under nitrogen at 55°-65° C. 1 mL of water saturated with the HPLC mobile phase and 2 mL of the HPLC mobile phase are added to the residue. After vortexing and centrifugation, a portion of the organic (top) layer is removed, and an aliquot is analyzed by HPLC on two 25 cm Partisil PXS 10/25 (Whatman) columns. The effluent is monitored by fluroescence with excitation set at 310 nm and emission at 440 nm.

The peak height is determined and the quantity of lasalocid is calculated from an external standard curve run on the same day. The fortification is done on acetonitrile enzyme deactivated liver sample because of lasalocid's instability on spiking into liver. Lasalocid is stable in livers from dosed animals.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. 80 mL glass centrifuge tubes.

b. Polytron: Brinkman Industries, equipped with small blending heat or equivalent.

c. 50 mL polypropylene tubes: Corning.

d. Centrifuge: Damon/IEC Model HN-s equipped with six-place fixed rotor or equivalent.

e. N-Evap, Model 111: Organomation Assoc., Inc., or equivalent.

f. Maxi Mix: Themolyne, Sybron Corp.

g. Pre-injection column: Whatman Partisil PXS 10/25 (10 micron micro-particulate silica, 25 cm, 4.6 mm id).

h. Analytical column: Two Whatman Partisil PXS 10/25 in series.

i. 100 μL syringe.

j. Sep-Pak silica cartridge: Waters Associates #51900, or equivalent.

k. Usual glassware.

2. Instrumentation

HPLC Chromatograph: Waters Associates, Model 244, or equivalent, with Schoeffel FS970 LC fluorometer, detector, Wisp 710B sample processor, and a strip chart recorder (Perkin Elmer Model 056 1001).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane—distilled in glass (Burdick and Jackson).

b. Methanol—distilled in glass (Burdick and Jackson).

c. Tetrahydrofuran—distilled in glass (Burdick and Jackson).

d. Acetonitrile—distilled in glass (Burdick and Jackson).

e. Ammonium hydroxide—ACS reagent grade.

f. Chloroform—distilled in glass (Burdick and Jackson), or equivalent.

g. HPLC mobile phase.

i. Prepare solvent mixture A, adding the components in order of decreasing volume percents:

(a) Hexane: 810 mL.

(b) Tetrahydrofuran: 150 mL.

(c) Methanol: 30 mL.

(d) Ammonium hydroxide: 10 mL.

ii. Mix thoroughly in separatory funnel and let stand until upper phase is clear (about 1 hr). Empty lower phase and discard. Reserve upper phase. Process three volumes (three separatory funnels) simultaneously.

The mobile phase is stable for a minimum of one week.

h. Preparation of water saturated with HPLC mobile phase.

i. Mix 50 mL water (deionized, distilled) with 100 mL HPLC mobile phase in a 250 mL separatory funnel. Shake vigorously for 30-40 sec.

ii. Let sit until lower layer (water) is clear. Remove lower layer immediately prior to use.

Prepare fresh each day.

DETERMINATIVE METHOD

D. STANDARDS

1. Source	Standard lasalocid (mw 612.80), Animal Health Research, Hoffman LaRoche.
2. Preparation of Standards	Preparation of lasalocid standard solutions for standard curve and sample fortification. a. 140 μg/mL lasalocid: Weigh exactly 72.66 mg lasalocid sodium salt standard into a 500 mL volumetric flask. Add THF to dissolve lasalocid and dilute to volume with THF. b. 70 μg/mL lasalocid: Dilute 50 mL of standard 2.a to 100 mL with tetrahydrofuran. c. 35 μg/mL lasalocid: Dilute 25 mL of standard 2.a to 100 mL with tetrahydrofuran. For standard curve, dilute 100 μL of each standard to 4 mL with mobile phase representing 1.4, 0.7, and 0.35 ppm respectively.
3. Storage Conditions	Store stock solutions in refrigerator at 4° C in stoppered volumetric flasks sealed with parafilm.
4. Shelf Life Stability	Solutions may be kept for five months. Each week, decant a fresh supply of each solution into screw-capped culture tubes, seal with parafilm, and store at room temperature for use in sample fortification and generation of daily standard curves.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Decontamination of Apparatus and Glassware

a. Meat grinder: All parts of the grinder that come in contact with the tissue sample should be washed with hot soapy water, rinsed, air-dried, rinsed with ehtyl acetate, and air-dried. The hot soapy water wash should be repeated between sets of samples expected to contain different levels of lasalocid.

b. Polytron: After homogenizing a sample, wipe the remaining liver off the shaft, using a clean paper towel, and rinse the shaft successively in water 2 × 50 mL (in a 250 mL centrifuge bottle), 50 mL ethyl acetate, and 50 mL acetonitrile, with polytron set at medium speed for 10-15 sec for each rinse.

2. Preparation of Samples

a. Weigh 10.0 ± 0.05 g of partially defrosted liver sample into a clean 80 mL glass centrifuge tube. Select a blank liver sample for a second control and three levels of fortification.

b. Add 40 mL of acetonitrile to each weighed sample and homogenize for 15-30 sec.

c. Fortify the homogenized blank samples according to the following table:

Fortification Level (ppm)	*Standard Lasalocid*	*µL of Fortifying Solution/10 g sample*
1.4	D.2.a	100
0.7	D.2.b	100
0.35	D.2.c	100

d. Homogenize each sample again for 15-30 sec.

e. Centrifuge for 10 min at 2000-2500 rpm.

f. Using a 25 mL graduated cylinder, transfer a 23.5 mL aliquot into a 50 mL screwcapped polypropylene tube. (This represents ½ of the sample extract, assuming 10 g liver to contain approximately 14 mL water.)

g. Add 20 mL hexane.

h. Shake vigorously for 15-20 sec, with venting.

i. Centrifuge at 1500-2000 rpm for 10 min at room temperature.

j. Aspirate hexane layer and discard.

k. To remove interfering fat, repeat steps g-j.

l. Evaporate acetonitrile layer to less than 0.5 mL under nitrogen using N-Evap at 45°-60° C. *Do not overdry.*

m. Add 5 mL chloroform and filter through Sep-Pak silica cartridge and evaporate to dryness under nitrogen using N-Evap at 45°-60° C. Do not overdry.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

n. Add 2.0 mL HPLC mobile phase and vortex for 15-20 sec with the screw cap on.

o. Transfer the mixture into 15 mL stoppered glass centrifuge tube with *tapered bottom*.

p. Centrifuge at 1500-2000 rpm for 10 min.

q. Transfer about 200 μL *clear* upper layer into a capped plastic vial for HPLC analysis.

r. With each set of samples, process a control liver, reagent blank, and a 0.7 ppm fortified liver to determine recovery.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. HPLC Analysis

a. Follow SOP to HPLC with the following exceptions:

   i. Do not filter or de-gas mobile phase.

   ii. Do not filter samples.

   iii. Each day, transfer only enough mobile phase mixture for a daily run from the amber gallon bottle into the HPLC solvent bottle. Cover solvent bottle with aluminum foil.

b. Set the fluorescence spectrophotometer as follows:

   Excitation: 310 nm Slit: 8 nm
   Emission: 440 nm Slit: 8 nm
   Sensitivity: 10, 3, or 1 as needed to keep lasalocid peak on scale

   PM gain: Norm
   Response: Norm
   Mode: Norm
   Zero Suppression: Off

c. Equilibrate the entire system with mobile phase until a stable baseline is obtained (about 30-45 min). If the column is new, equilibrate with mobile phase until a constant retention time and fluorescent response for lasalocid are obtained (about 24 hr).

d. Measure the flow rate at the beginning of each day's samples and recheck periodically throughout the day.

e. Using Wisp 710B sample processor, inject (in duplicate) 20 μL portions each of sample extracts and mixed standards.

f. Retention time for lasalocid is about 6.5 min at a flow of 2.0 mL/min. Chart speed should be about 0.5 cm/min. Allow 10 min between injections.

g. With each set of samples, inject three different lasalocid standards before the set, and the same three standards after the set. The concentration of lasalocid injected should be above, below, and approximately the same concentration as expected to be present in the final dilution of the samples to be assayed.

ppm Lasalocid in Liver	*Expected Concentration Lasalocid per Final Dilution of Liver Extract*
0.35	35 μg/mL
0.7	70 μg/mL
1.4	140 μg/mL

h. If the HPLC system is not to be used for 8 hr or more, flush the entire system with hexane.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

2. Automatic Integration Devices	Laboratory instrumentation that automatically provides peak area or peak heights can be used in the method. The technique used should be common for all the samples and the calculation should reflect techniques used in height or area.
3. Retention Time or Volume	a. The retention volume or time will shift based on the history of the column. The standards are run at the start and end of a series and are used to determine responses as well as retention time. b. Variation in retention time is also influenced by the time-marking process, either manual or automatic. This also is resolved by inspection of multiple standards.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure	Calculation of lasalocid in sample: Using area data or peak height from 1.40, 0.70, and 0.35 ppm standard lasalocid injections, construct a linear standard curve based on the formula $y = mx + b$, where x is the peak area or height and y is ppm. Calculate regression coefficient and standard error of estimate for these data. Calculate recovery from a spiked sample included in every set. Correct sample values for recovery.
2. Reference	NADA 96-298V, Hoffman LaRoche.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Lasalocid in Bovine Liver		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Hexane Methanol Tetrahydrofuran Acetonitrile Ammonium hydroxide	These solvents are typically very flammable or corrosive. The vapors are extremely irritating to the skin, eyes, and respiratory system.	These solvents should only be handled in an efficient fume hood. Excepting acetonitrile, the other solvents are used to prepare the HPLC mobile phase. This may be done in a fume hood away from any heat-producing device.
4. Disposal Procedures	Organic solvent mixtures	See above	The large volume may be reduced by partial evaporation in a fume hood and the remaining solvent can be stored in waste cans until disposed of by the contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability % CV*
Lasalocid	0.025-1.4	80-110	≤ 10

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	10.0 g ± 0.05 g
b. Acetonitrile volume	40.0 mL
c. Blend time	20 sec ± 5 sec
d. Volume transferred	23.5 mL
e. Evaporation	No overdrying
f. Vortex time	20 sec ± 5 sec
g. Upper layer	Clear

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—0.35, 0.70, 1.40 ppm. Standard curves prepared on three separate days.

ii. Phase II: Duplicate recoveries (self-fortified samples) on three separate days on blanks and at three different levels (each day) within the analytical range, 0.35-1.40 ppm.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples submitted by FSIS Science & Technology Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this analysis.)

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents:

i. Initially, at least one check sample biweekly per analyst.

ii. Random replicates or blind check samples chosen by the supervisor and/or the Laboratory QA Officer.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) CUSUM control chart on differences between replicates.

(c) All percent recoveries.

(d) For all recoveries—the running average, standard deviation, and coefficient of variation on last acceptable 10.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Liver.

NOTE: Method has been extended to poultry fat and skin.

b. Sample receipt size: Varied; enough to obtain matrix required for all quantitative and confirmation tests and reserve sample.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: ≤ 1 yr.

ii. Condition: Frozen.

6. Sample Set

a. Blank tissue (control blank).

b. Blank tissue fortified at 0.7 ppm.

c. Samples processed the same day.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

7. Sensitivity	a. Lowest detectable level (LDL): 0.025 ppm.
	b. Lowest reliable quantitation (LRQ): 0.35 ppm.
	c. Minimum proficiency level (MPL): 0.35 ppm.

LYSERGIC ACID DIETHYLAMINE AND PHENCYCLIDINE

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. [Reserved]
E. Extraction Procedure 4
F. [Reserved]
G. [Reserved]
H. Hazard Analysis 5
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The drugs of interest are extracted from an alkaline tissue sample with ethyl acetate. The organic phase is then shaken with dilute hydrochloric acid. The pH of the acid is adjusted with base and extracted with chloroform. The chloroform layer containing the drugs is then removed and spotted on two separate TLC plates, developed, and visualized with the appropriate spray reagents.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

An equivalent can be substituted for any apparatus listed below if necessary.

a. 50 mL polypropylene screw-top disposable centrifuge tubes.
b. Mechanical horizontal shaker (240 cycles/min).
c. Vortex test tube mixer.
d. Centrifuge capable of 3000 rpm.
e. Lab timer.
f. 15 mL glass centrifuge tube.
g. Water bath, 15-20° C.
h. Repipet dispensers, 5 mL and 30 mL.
i. 10 mL class A volumetric flasks.
j. 100 mL volumetric flasks.
k. 100 μL syringe (glass).
l. E. Merck 60F-254 silica gel plates.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Ethyl acetate—distilled in glass.

b. 50% NaOH—reagent grade.

c. Granular sodium sulfate.

d. 0.5N HCl—reagent grade.

e. Chloroform—distilled in glass.

f. LSD (lysergic acid diethylamide)—0.02 mg/mL in ethyl acetate.

g. PCP (phencyclidine HCl)—0.117 mg/mL in ethyl acetate.

h. Acetone—distilled in glass.

i. Methanol—distilled in glass.

j. Iodoplatinate (potassium)—for alkaloids and other organic nitrogen compounds. Mix 3 mL 10% hexachloroplatinic (IV) acid solution with 97 mL water and add 100 mL 6% potassium iodide solution in water. Freshly prepare the reagent before use.

k. Dimethylaminobenzaldehyde-hydrochloric acid—for indole derivatives. Dissolve 1 g 4-dimethylaminobenzaldehyde in 50 mL 36% hydrochloric acid and add 50 mL ethanol.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

a. Add 1 mL of 50% sodium hydroxide to 10 g of homogenized meat in a 50 mL polypropylene centrifuge tube.

b. Add 5 g sodium sulfate and 30 mL ethyl acetate to the mixture.

c. Shake the solution on a horizontal shaker for 10 min.

d. Centrifuge the tube at 3000 rpm for 5 min.

e. Decant the ethyl acetate upper layer into another 50 mL polypropylene centrifuge tube.

f. Remove and discard any oily residue that transfers over with a Pasteur pipet.

g. Add 5 mL of 0.5N hydrochloric acid, shake for 3 min using a horizontal shaker, and centrifuge at 2000 rpm for 3 minutes.

h. Aspirate and discard the ethyl acetate (upper layer).

i. Transfer the 5 mL of 0.5N hydrochloric acid solution to a 15 mL graduated glass centrifuge tube.

j. Cool the solution in a water bath for 5 min and add 1 mL of a 50% NaOH solution; cool again for 3 min.

k. Agitate by vortexing for 30 sec.

l. Cool the solution again in a water bath for 2 min.

m. Add 100 mL of chloroform to the solution (alkaline); shake manually for 3 min.

n. Centrifuge for 5 min at 200 rpm.

o. Aspirate and discard half of the aqueous alkaline layer:

   i. With a 100 μL syringe, remove two 40 μL aliquot portions of the chloroform layer and spot separately on different silica gel plates. Spot a detectable amount of LSD and PCP separately on each plate.

   ii. Develop the plates in the following solvent system—acetone:methanol (80:20). Saturate the tank prior to use.

   iii. After development, air dry the plates and spray for LSD using P-dimethylaminobenzaldehyde. Spots will be violet on a yellow plate. Spray for PCP using iodoplatinate. Spots will be brown on a yellow plate.

   NOTE: Spray lightly—do not overspray.

   Rf LSD—0.33; Rf PCP—0.25.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Analysis of Lysergic Acid Diethylamide and Phencyclidine in Tissue.

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
a. Ethyl acetate e. Chloroform h. Acetone i. Methanol	These organic reagents are very flammable and corrosive and the vapors are extremely irritating to the skin, eyes, and respiratory system.	These reagents should only be used in an efficient fume hood away from any heat-generating devices.
b. 50% NaOH	Caustic, may cause irreversible skin damage.	Flush immediately with water if any spattering occurs.
E. Extraction		
o. (iii) Spray for LSD, PCP	Acidic vapors	Spray in fume hood and use some form of a cardboard partition to trap the deflected spray.

4. Disposal Procedures

Organic solvent mixtures	See above	Segregate chlorinated from nonchlorinated and hold in designated storage cans until disposed of by the contractor or in-house specialist.
Extracted tissue waste	Harmful vapors	Allow organic vapors to dissipate in the fume hood and then store waste in a plastic bag awaiting disposal.

CYROMAZINE AND MELAMINE

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 10
G. Calculations 11
H. Hazard Analysis 12
I. Worksheet 13
J. Quality Assurance Plan 17

II. Confirmatory Method

A. Introduction 19
B. Equipment 20
C. Reagents and Solutions 21
D. Standards 22
E. Extraction Procedure 23
F. Analytical Confirmation Procedure 25
G. [Reserved]
H. Hazard Analysis 28
I. [Reserved]
J. Quality Assurance Plan 29

I. DETERMINATIVE METHOD

A. INTRODUCTION

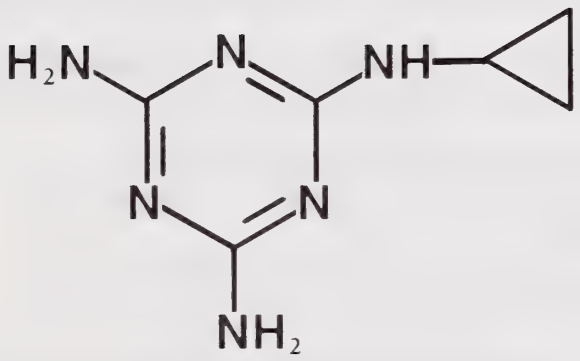
1. Theory and Structure

Cyromazine and melamine are extracted from tissue samples using acetonitrile/water, followed by a cleanup through a C_{18} Solid Phase Extraction (SPE) device.

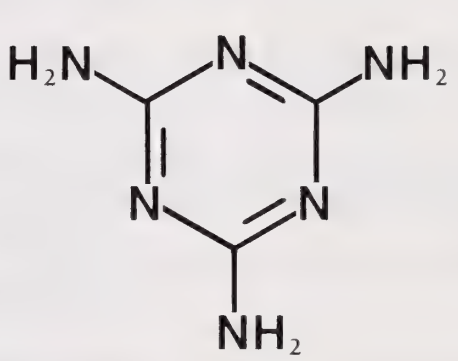
They are then quantitated by High Performance Liquid Chromatography (HPLC) equipped with an amine column and UV detector. This procedure is a modification of the Ciba-Geigy AG-417A method.

Cyromazine: $C_6H_{10}N_6$
F.W. = 166.18

Melamine: $C_3H_6N_6$
F.W. = 126.13



CYROMAZINE



MELAMINE

2. Applicability

This method has been evaluated for cyromazine and melamine residues analysis in poultry, red meat tissues, and processed products (ref., *J. Agric. Food Chem.*, 1988, 36, 1009-1012).

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted for any apparatus listed below if necessary.

a. Bottles, milk dilution, 160 mL (Corning 1368). A 16-oz wide-mouth French square bottle may be optionally used if larger sample is to be used, i.e., 25 g.

b. Column, Econo-column, polypropylene, 0.7 × 4 cm volume (Bio-Rad #731-1110).

c. Test tube, Teflon-lined screw-cap (Corning 9825 and 9998).

d. Vacuum flask, 1 L.

e. A Vac Elut SPE Processing Station (Analytichem AI 6000).

f. Graduated cylinders, 50 mL and 100 mL.

g. Polytron homogenizer (Brinkman Instruments).

h. N-Evap (Organomation Associates Inc., model 11).

i. C_{18} syringe barrel, type SPE (J. T. Baker #7020-3) and 75 mL reservoirs and adaptors (Analytichem #607500 and 636001 respectively).

j. Syringe glass, 60 mL, with Luer tip.

k. Vortex mixer.

l. Optional repipets.

m. Optional 14-gauge stainless steel cannula for pipetting 50 mL aliquot with glass syringe.

2. Instrumentation

HPLC: Waters, Model WISP 712 equipped with sample injector and automated pump (Model 6000A). A column switching valve (Waters Model 60057) is an advisable optional component.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Acetic acid, 1N, reagent grade.

b. Acetonitrile, HPLC grade.

c. Water, HPLC grade or at least 10 megohm.

d. Methanol, HPLC grade.

e. Ammonium hydroxide, reagent grade.

f. Sodium hydroxide, 1N aqueous, reagent grade.

g. Hydrochloric acid, 0.1N aqueous, reagent grade.

h. Acetonitrile/water, 90% v/v: Add 900 mL of acetonitrile to a 1000 mL volumetric flask and dilute to the mark with water.

i. Acetonitrile/water, 95% v/v: Add 950 mL of acetonitrile to a 1000 mL volumetric flask and dilute to the mark with water.

j. Ammonium hydroxide/methanol, 5% v/v: Add 5 mL of ammonium hydroxide to a 100 mL volumetric flask and dilute to volume with methanol.

k. Ammonium hydroxide/methanol, 25% v/v: Add 25 mL of ammonium hydroxide to a 100 mL volumetric flask and dilute to volume with methanol.

l. Methanol/water, 50% v/v: Add 50 mL of methanol to a 100 mL volumetric flask and dilute to volume with water.

m. Methanol/water, 90% v/v: Add 90 mL of methanol to a 100 mL volumetric flask and dilute to volume with water.

n. Resin 1, Dowex 50W-X4 Cation Exchange Resin, 200-400 mesh, Bio-Rad.

o. Resin 2, Dowex 1-X8 Anion Exchange Resin, 50-100 mesh, chloride form. (Refer to section E for column preparation.)

p. Resin 3, Dowex 50W-X4 Cation Exchange Resin, 50-100 mesh, Bio-Rad.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Cyromazine (96.5%) and melamine (99.0%) standards can be purchased from:

Ciba-Geigy Corp.
410 S. Swing Road
Greensboro, NC 27049
Telephone: (919) 292-7100

2. Preparation of Standards

a. Stock standard: 1 mg/mL.

i. Cyromazine: Place 50 mg of the standard in a 50 mL volumetric flask. Dilute to volume with methanol.

ii. Melamine: Place 50 mg of the standard in a 50 mL volumetric flask. Dilute to volume with 50% methanol/water.

b. Working standard: 10 μg/mL.

i. Cyromazine: Add 1 mL of the stock standard (2.a.i.) to a clean 100 mL volumetric flask and dilute to volume with methanol.

ii. Melamine: Add 1 mL of the stock standard (2.a.ii.) to a clean 100 mL volumetric flask and dilute to volume with 50% methanol/water.

c. HPLC standards.

Dilute the working standards (2.b.) with the mobile phase as follows:

Cyromazine/Melamine Standard mL of 10 μg/mLtandard	*Final Volume (mL)*	*Final Concentration ng/mL*
0.5 mL	100	50
1.0 mL	100	100
2.0 mL	100	200

3. Storage Conditions

All standards are to be kept tightly closed in a glass-stoppered bottle and stored at 4° C when not in use.

4. Shelf Life Stability

Stability is based upon the storage conditions listed in section D.3.

a. Stock standards: One year.

b. Working standards: Six months.

c. HPLC standards: Three weeks.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Column Preparation (for resin 2)

a. In a large beaker, swirl about 100 g of Dowex 1-X8 resin, 50-100 mesh, chloride form, with about 500 mL of 1N acetic acid.

b. Allow the resin to settle and decant the supernatant liquid together with the suspended fines.

NOTE: Steps b, c, and e may be facilitated with the use of a sintered glass filter.

c. Wash the resin with 500 mL distilled water as in steps a and b.

d. Wash the resin twice with 1N sodium hydroxide. The resin will darken in color.

e. Wash the resin with several portions of distilled water until the supernatant is about neutral or the same pH as the distilled water.

f. Transfer a slurry of the resin into an Econo-Column sufficient to prepare a 2 mL bed.

g. Prewash the 1-X8 resin column with 10 mL distilled water before use. This column is ready to use in section E.3.

2. Sample Extraction

a. Weigh a 10 g sample of tissue into a 160 mL square milk dilution bottle. Optionally, a 25 g sample may be weighed into a 16 oz wide-mouth French square bottle.

b. Weigh two 10 g portions of tissue blank. Label them as follows:

i. Control blank tissue.

ii. Control fortified tissue.

Fortify the blank tissue by adding 5 mL of the HPLC standard solution (section D.2.c.). This will yield a recovery of 100 ng/g.

c. Add 92 mL of 90% acetonitrile/water to the bottle and homogenize for 1 min at a power setting of 6-8 and allow to settle. For the optional 25 g sample, use 230 mL of 90% acetonitrile/water.

d. Place the sample in a freezer for at least two hours. Warm to room temperature. A clear acetonitrile/water layer will form on the top. Filter the top layer through glass wool. Discard the bottom layer. Extract may be centrifuged, but should not be filtered through filter paper. Some filter papers contain melamine from the manufacturing process.

e. Load an Econo-Column with a 2 mL bed of resin 1, Dowex 50W-X4. Add about 5 mL of water to the column and place a perforated cap on the column. Insert the Luer tip of a C_{18} SPE column in the Econo-Column cap; add a few mL of 90% acetonitrile/water to the SPE to prime. Place the SPE adaptor on the SPE and add the syringe barrel reservoir.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

f. Add a 50 mL aliquot of the sample to the reservoir. This is measured with a glass syringe or a graduated cylinder. 50 mL represents a 5 g sample.

g. Allow the sample extract to flow through the SPE Econo-Column assembly either by gravity or with vacuum assistance. Flow rate should not exceed 2 drops a second. Discard this liquid.

h. Remove the SPE column and reservoir from the assembly and connect a clean reservoir to the Econo-Column cap.

i. Wash the ion-exchange column with 50 mL of 0.1N HCl. Discard the wash. Vacuum may be used.

j. Wash the ion-exchange column with 50 mL of 90% methanol/water. Discard the wash. Vacuum may be used.

k. Wash the ion-exchange column with 10 mL of methanol. Discard the methanol. Vacuum may be used.

l. Elute the cyromazine/melamine from the ion exchange resin with 20 mL of 5% ammonium hydroxide/methanol into a 50 mL screw-cap test tube. This step must be done in a fume hood.

m. Evaporate the eluate to dryness using air in the N-Evap (40° C bath). This step must be done in a fume hood.

3. Sample Cleanup

a. Fit the Dowex 1-X8 column, resin 2, prepared in section E.1, into the top cap of a second Econo-Column containing a 2 mL bed of Dowex 50W-X4 resin (50-100 mesh), resin 3. The bottom column must be primed with about 5 mL of distilled water prior to connecting these columns together to avoid an air lock.

b. Dissolve the sample from step 2.m. in 10 mL of distilled water and transfer to the upper anion column (resin 2) either directly or using a reservoir.

c. Rinse the sample tube with another 10 mL of distilled water and add to the top column or reservoir. Repeat again with 5 mL of distilled water (total of 25 mL of water).

d. After the aqueous has eluted through the top column, remove this column. Discard this wash.

e. Rinse the Dowex 50W-X4, 50-100 mesh (bottom column, resin 3), with 10 mL of HPLC grade methanol. Discard this wash.

f. In a hood, elute the cyromazine and melamine, using 30 mL of 25% ammonium hydroxide/methanol. This is accomplished by using a syringe barrel reservoir or by making three 10 mL additions of the eluent directly to the open column.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

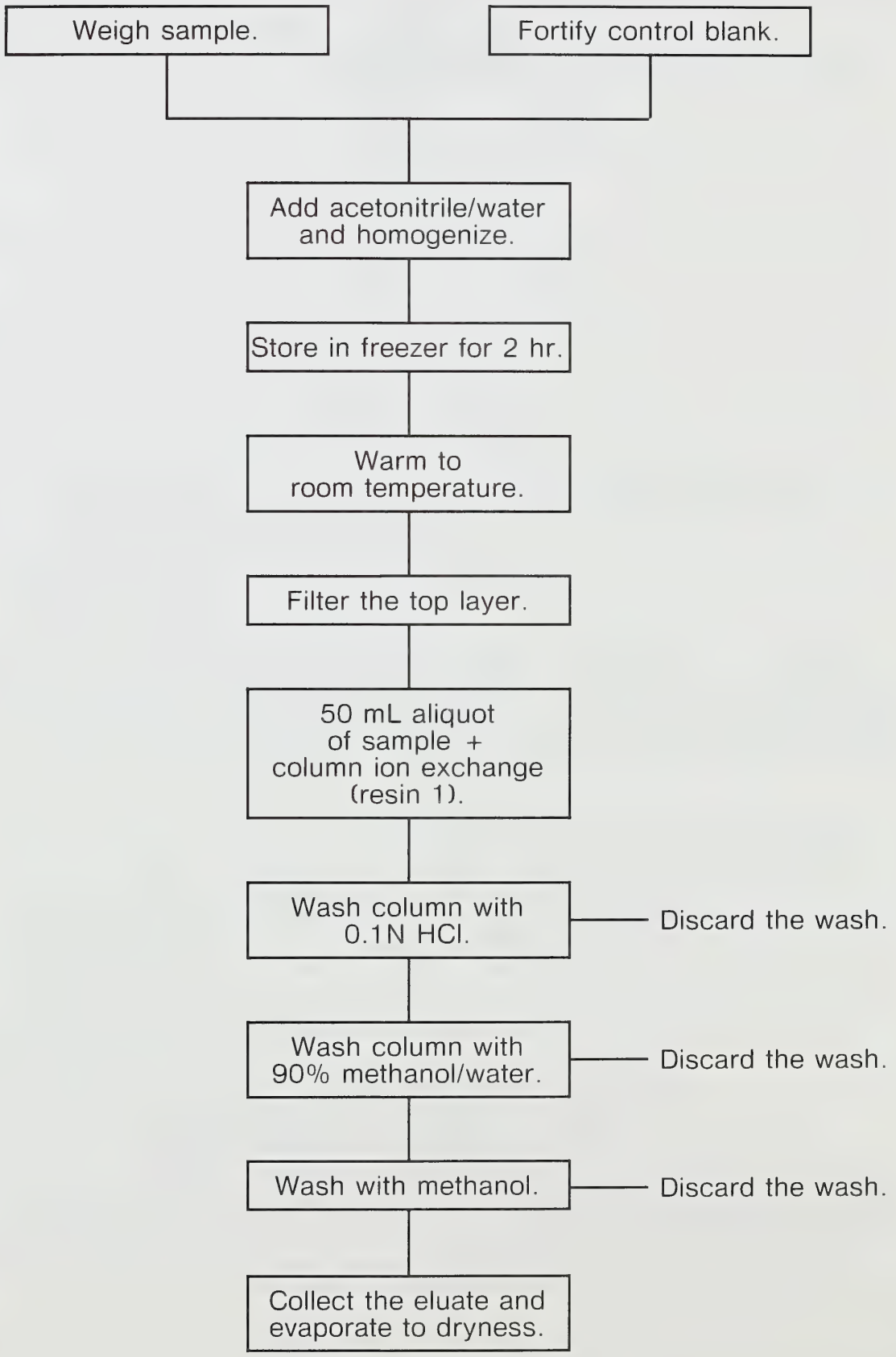
g. Collect the eluate in a screw-cap test tube and evaporate as in step 2.m.

h. Dissolve the dried sample in 2.5 mL of mobile phase or 90% acetonitrile/ water and filter through a Nylon 66 HPLC 0.45 micron filter. Sample is now ready for analysis.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

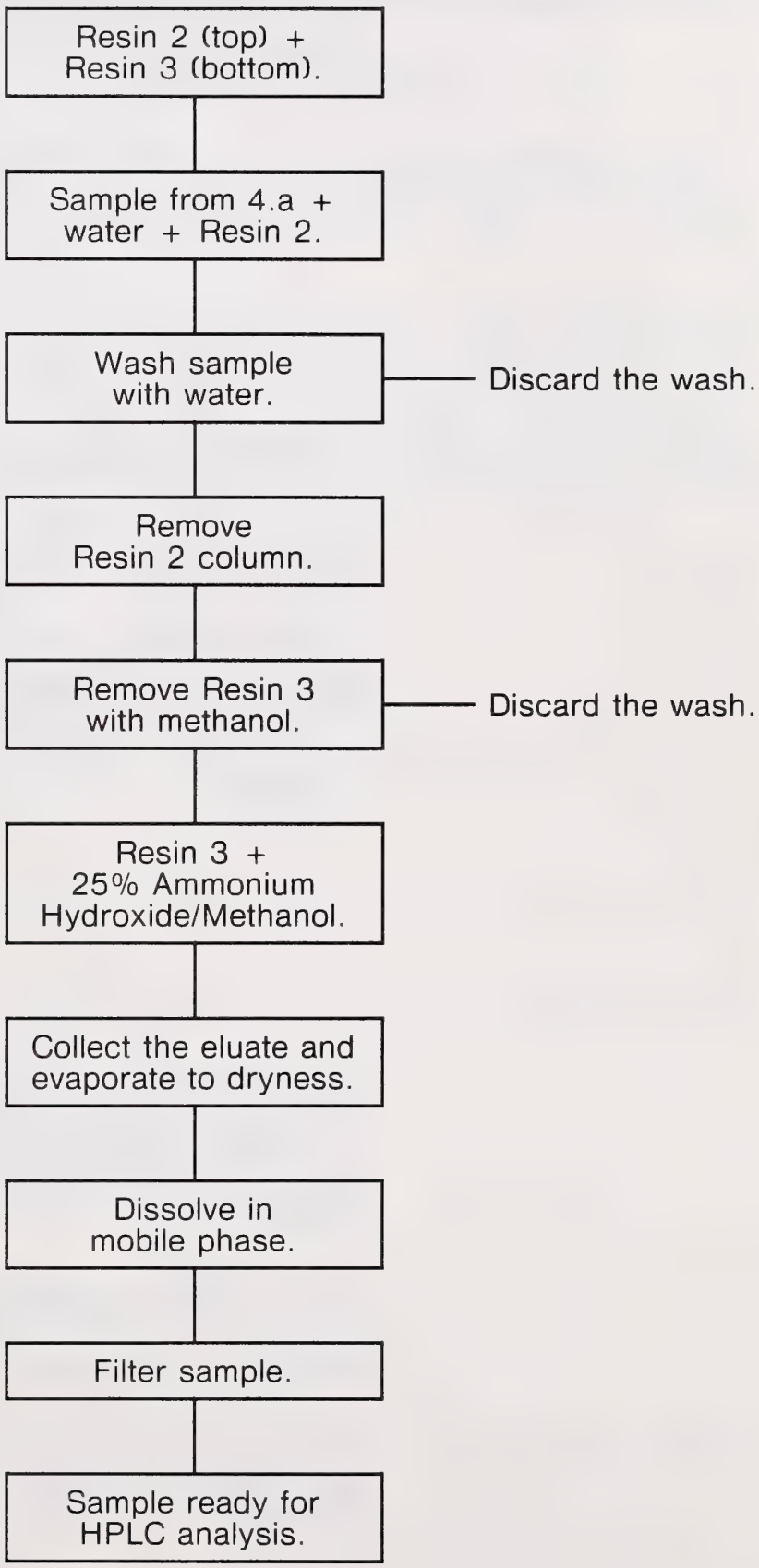
4. Flow Chart Summary a. Sample extraction.



I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Sample cleanup.



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

The following parameters may be modified as necessary to compensate for daily variations in instrument performance characteristics.

a. Mobile phase	95% acetonitrile/water. Because dissolved oxygen absorbs UV at 214 nm, the mobile phase is sparged with helium during long automated runs.
b. Flow rate	1.0 mL/min, using 4.6 mm diameter HPLC column. Rate is adjusted down when using microbore column.
c. Attenuation	0.005 AUSF or as needed.
d. Detector	UV detector.
e. Wavelength	214 nm.
f. Injection volume	0.025 mL.
g. Chart speed	0.5 to 1.0 cm/min.
h. Approximate retention time (for a two-column system)	Cyromazine: 10.2 min. Melamine: 14.9 min.
i. Column	Hibar 250-4 pre-packed with 5 micron NH_2. It has been found that frequently a single 250 mm column does not give adequate separation tissue substances. Normally the chromatography is done using two 5 micron NH_2 columns such as Lichrosorb #50376.

NOTE: Because of the excesive times needed to elute some of the late-eluting interfering tissue substances, it is helpful to use a column switching valve in the system so that after the compounds of interest have been eluted onto the second column, the first column may have its flow reversed and pumped to waste using a second pump while the chromatography continues through the second column.

NOTE: Waters C_{18} Sep-Packs may be used in place of the syringe barrels; however, they must be attached to 75 mL reservoirs. The units are then suspended over the reservoirs, which are connected to the caps of the Dowex 50W-X4 200-400 mesh Econo-Column (resin 1).

2. Known Interferences

Processed products can give background peaks that obscure peaks of interest. False positives can occur. GC/MS confirmation of HPLC positive sample is essential.

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Standardize the HPLC by injecting 25 μL of the standards in section D.2.c under the conditions stated in section F to give a standard curve of 1 to 4 ng on 4.6 mm diameter columns.

b. Determine the peak heights or areas of the injected standards.

c. Using the peak data (height or area) and the respective injected amounts of the standard, calculate the least squares standard curves; alternatively, construct the standard curve by plotting peak height vs. standard concentration.

d. Inject an aliquot of the sample extract from section E.3.h into the HPLC using the same conditions as those for the standards. Determine the cyromazine and melamine in the samples by calculation, using the least squares regression on the peak data from the samples or by reading the values from constructed curves. Recovery corrected values are then obtained by dividing the resulting values by respective fractional recoveries from a spiked sample that has been carried through the analysis.

$$\text{ppb cyromazine} = \frac{\text{ng cyromazine found / Rc}}{\text{g tissue equivalent injected}}$$

$$\text{ppb melamine} = \frac{\text{ng melamine found / Rm}}{\text{g tissue equivalent injected}}$$

$$\text{ng cyromazine/melamine found} = \frac{\mu g}{mL} \times \text{final volume (mL)} \times \text{d.f.}$$

where:

Rc and Rm are the fractional recoveries for cyromazine and melamine respectively.

If needed, the factor 1.317 times the melamine value gives the cyromazine equivalent of the melamine.

μg/mL = μg/mL obtained from standard curve.

d.f. = sample volume/aliquot taken.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Analysis of Cyromazine and Melamine Residues in Animal Tissues

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Acetic acid Hydrochloric acid	Flammable, burn skin upon contact. Ingestion may cause severe corrosion of mouth and G.I. tract. Eye irritation.	Use under well-ventilated hood. Avoid contact with skin, eyes.
Sodium hydroxide Ammonium hydroxide	Very corrosive. Ingestion will result in vomiting, collapse. Will generate extensive amount of heat if solution is made up from bulk.	Same as above.
Acetonitrile	Poisonous, flammable, may cause skin irritation.	Same as above.
Methanol	Flammable.	Same as above.
D. Standards		
Melamine Cyromazine	Toxic by ingestion. Causes skin and eye irritation upon contact.	Same as above.

4. Disposal Procedure

Acetic acid Hydrochloric acid Ammonium hydroxide Sodium hydroxide	See above.	Flush down sink with lots of water. Use separate sink for acid vs caustic.
Organic solvent mixture/standard	See above.	Segregate chlorinated from nonchlorinated material before disposal by the contract or in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Cyromazine/Melamine*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

CYROMAZINE / MELAMINE

Set _____

Analyst _____

Date Start _____

Date Finish _____

2 X Running Average Recovery

Cyromazine _____ Melamine _____

Standard Curve Regression Analysis for y = mx + b

	Cyromazine	Melamine
m		
b		
r		

		CALCULATION CONTROL POINTS				CYROMAZINE			MELAMINE		
Vial #	Sample Identification	Sample Wt (g)	ACN Added (mL)	Aliquot Taken (mL)	Final Volume (mL)	Peak Ht (cm)	ppb Found	ppb Actual	Peak Ht (cm)	ppb Found	ppb Actual
	Blank Direct										
	1/2 X Direct										
	1 X Direct										
	2 X Direct										
	2 X Recovery										

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability %CV*
Cyromazine	25-100	60-115	≤ 20 at 50 ppb
Melamine	25-100	40-115	≤ 30 at 50 ppb

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	10 g ± 0.1 g
b. Aliquot taken	50 mL
c. Final volume	2.5 mL
d. Flow rate of SPE column	≤ 2 drops/sec
e. Evaporation temperature	40° C
f. pH of anion exchange resin supernatant	7.5 ± 0.5

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Triplicate standard curve on each of the cyromazine and melamine standards.

(a) Blank.

(b) 50 ng/mL.

(c) 100 ng/mL.

(d) 200 ng/mL.

ii. Phase II: Fortified samples—Duplicate analyses on fortified samples at 0, 25, 50, and 100 ppb on three consecutive days, one set each of red meat and poultry.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples submitted by supervisor, with 7 samples per species (beef and poultry) fortified at least at 50 ppb for poultry and 100 ppb for beef. A blank tissue must also be included.

(b) Report data to Chemistry Division.

Notification from Chemistry Division is required to commence official analyses.

b. Acceptability criteria.

Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst containing both cyromazine and melamine using different species. At least one out of four of these samples is to be confirmed by mass spectrometry.

ii. Random replicates chosen by supervisor or Laboratory QA Officer.

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on percent difference between replicates.

(c) All % recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Red meat and poultry muscle.

b. Sample receipt size, minimum: 30 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage:

i. Time: 6 months.

ii. Condition: –20° C.

6. Sample Set

Each set must contain a reagent blank, a blank tissue, a blank tissue fortified at 50 ppb (for poultry) and 100 ppb (for beef) and samples that will be processed on the same day.

7. Sensitivity

a. Lowest detectable level (LDL): 25 ppb.

b. Lowest reliable quantitation (LRQ): 50 ppb (poultry), 100 ppb (beef).

c. Minimum proficiency level (MPL): Not determined.

II. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory	This method confirms the presence of TMS derivatives of cyromazine and melamine residues extracted from poultry and red meat tissues from the determinative method. The TMS derivatives are formed by heating the residue with BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) in the presence of TMCS (trimethylchlorosilane).
2. Applicability	Refer to Determinative Method, section A.2.

II. CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus	a. Temp-block module heater, Lab-Line Instruments, Inc., or equivalent. b. Sand for 1.a. c. Conical centrifuge tube with stoppers, 15 mL. d. Volumetric flasks, 10 mL and 200 mL. e. Syringe, 250 μL.
2. Instrumentation	GC/MS: Hewlett Packard 5970B MSD/5890 GC equipped with 30 m DB5 column (J & W Scientific, Inc.), 0.25 mm id, 0.25 micron film thickness.

II. CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Refer to Determinative Method, section C.

b. Acetone: Fisher Optima Grade or equivalent.

c. Acetone/Methanol, 50% v/v: Mix 250 mL each of acetone and methanol in a clean 500 mL volumetric flask.

d. Acetonitrile: Stored in molecular sieve as drying agent.

e. Trimethylchlorosilane (TMCS): Pierce Chemical Co., catalog #88530.

f. N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% TMCS: Pierce Chemcial Co., catalog #38831.

II. CONFIRMATORY METHOD

D. STANDARDS

1. Source	Refer to Determinative Method, section D.1.
2. Preparation of Standards	a. Stock standard. i. Cyromazine stock: 1 mg/mL. Prepare by dissolving 10 mg of the compound in 10 mL of 50% acetone/methanol, v/v. ii. Melamine stock: 0.05 mg/mL. Prepare by dissolving 10 mg of the compound in 200 mL of 50% acetone/methanol, v/v. b. Working standard: 1 μg/mL. i. Cyromazine standard: Dilute 10 μL of the stock standard (a.i) into 10 mL volumetric flask and bring up to volume with acetone. ii. Melamine standard: Dilute 200 μL of the stock standard (a.ii) into 10 mL volumetric flask and bring up to volume with acetone.
3. Storage Conditions	Refer to Determinative Method, section D.3.
4. Shelf Life Stability	Refer to Determinative Method, section D.4.

II. CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE

1. Derivatization

Refer to determinative method, section E, for column preparation and sample extraction.

a. Prior to derivatization of the sample, the analyst should verify that the process is functioning. This can be accomplished by derivatizing approximately 100 μL of 1 ppm working standards prepared in section D.

b. Place the HPLC eluate containing the cyromazine and melamine in a 15 mL conical centrifuge tube. The eluate should represent at least 4 g of 40% of tissue sample.

c. Evaporate the eluate to dryness under a stream of nitrogen at about 50° C.

d. Add 100 μL of acetonitrile and mix for 30 sec with a vortex mixer.

e. Add 100 μL of BSTFA wtih 1% TMCS.

f. Mix for 30 sec and heat at 110° C for 1 hour in a sand bath.

NOTE: This heating will result in the cyromazine derivative with the following ions: 171, 181, 295, and 310. Prolonged heating or heating at a higher temperature will result in the formation of the trisubstituted cyromazine derivative with characteristic ions at 171, 253, 269, 283, 367, and 382. Whether it is more advantageous to drive the reaction to completion and monitor the trisubstituted cyromazine has yet to be determined.

g. Cool the sample to room temperature and put it in the freezer (approximately −20° C) for 20 min.

h. Immediately discard the lower clear liquid organic layer (approximately 100 μL).

i. Inject 2.5 μL of the remaining sample, using 0.5 μL acetonitrile plug. Alternatively, directly inject 2.5 μL of the upper organic layer in the bilayer sample in step h above (before discarding the lower organic layer).

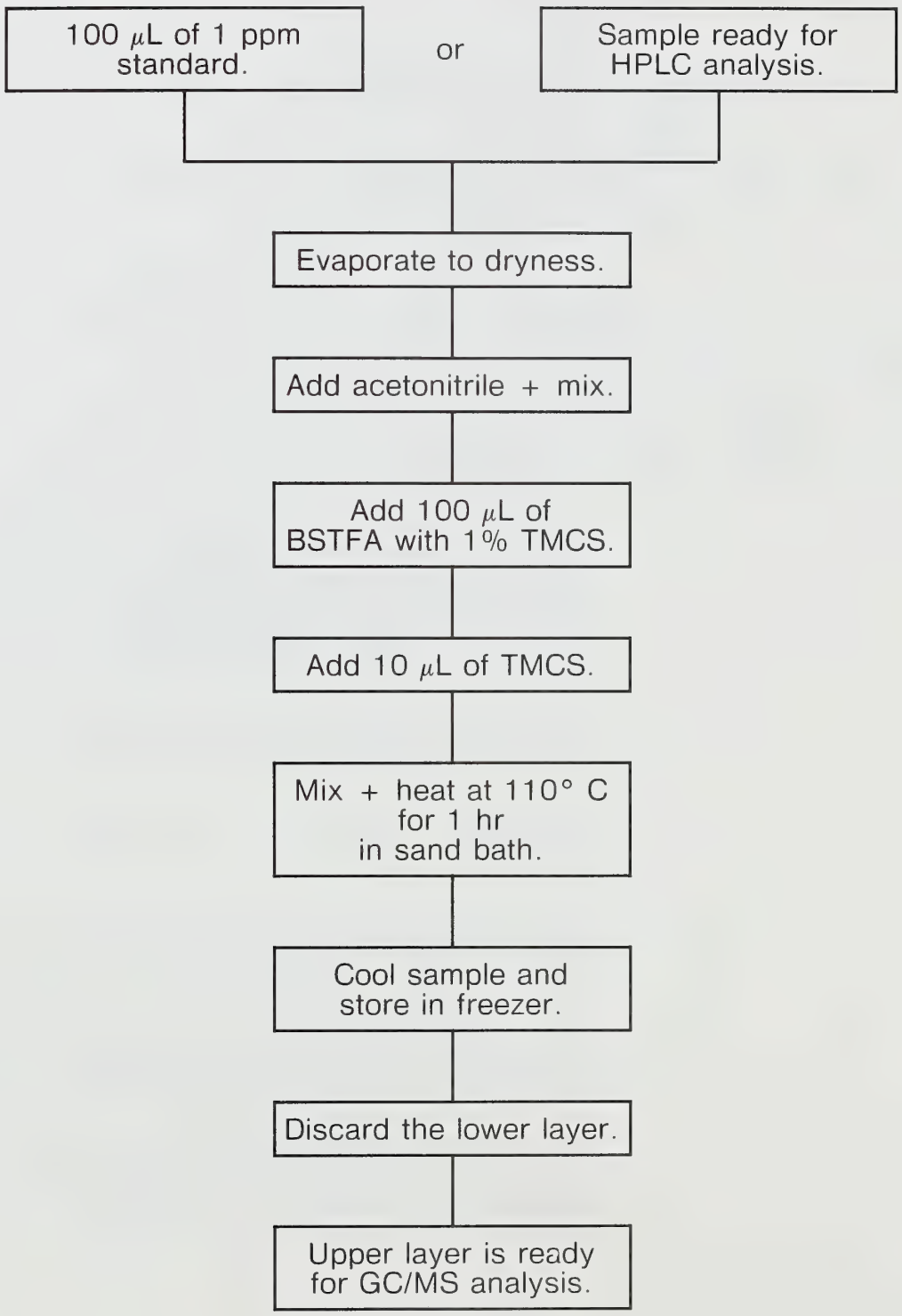
j. Run approximately 100 ng each of the cyromazine and melamine standards concurrently with the sample by adding 100 μL of 1 ppm working standards into a vial and performing steps b through i above.

k. Run a method blank and recovery standard concurrently with the sample by performing steps b through i above. The recovery standard concentration should be within the same range as the concentration expected for the sample.

II. CONFIRMATORY METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary Sample derivatization.



II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions and parameters.

Detector	MS-EI-SIM.
Detector temperature	180-250° C (MS source temperature).
Injection mode	Splitless.
Injection temperature	260° C.
Carrier gas	Helium.
Flow rate	Approximately 30 cm/sec.
Column head pressure	10 psi.
Temperature program	
Initial temperature	100° C.
Hold time	1 min.
Program rate	15°/min.
Final temperature	250° C.
Final hold time	9 min.
Transfer line	270° C.
Splitless injection	1 min vent delay.

b. Mass spectrometer conditions.

Analyzer parameters	Set by autotune program.
Electron energy	70 ev.
Electron multiplier	200-600 v above the autotune voltage.

NOTE: Parameters for Selected Ion Monitoring (SIM)—Set up two separate retention time windows and monitor four ions within each window using 150 msec dwell time per ion.

- Cyromazine—m/z 171, 181, 295, 310.
- Melamine—m/z 171, 197, 327, 342.

2. Required Samples

a. Inject 2.5 μL of standard to check the performance of the instrument.

b. Inject 2.5 μL of acetonitrile to check carryover.

c. Inject 2.5 μL of method blank to check background interferences.

d. Inject 2.5 μL of recovery standard.

e. Inject 2.5 μL of the sample.

NOTE: if the instrument has been performing well, steps 2.a and 2.b can be omitted.

NOTE: The signal to noise ratio of the least abundant ion for either cyromazine or melamine in the recovery standard should at least be equal to 5.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

a. All four ions for each analyte must be present.

Compound	*Ions Monitored*
	(+) = Base ion
Cyromazine	171, 181, 295(+), 310
Melamine	171, 197, 327(+), 342

b. At least two ratios, relative to the most intense ion, must be within 15% of those found in the recovery sample spiked at the level found in the suspect sample. Typical ratios and retention times:

Compound	*Relative Retention Time (min)*	*Ions*	*Relative Ratio*
Cyromazine	11.38	171/295	0.416
		181/295	0.482
		310/295	0.816
Melamine	10.54	171/327	0.629
		197/327	0.135
		342/327	0.608

c. Retention time must agree within 5 seconds of that found for the fortified sample.

Compound	*Retention Time (min)*
Cyromazine	11.38
Melamine	10.54

d. Blank should show no interferences.

e. Postulated structure of confirming ions.

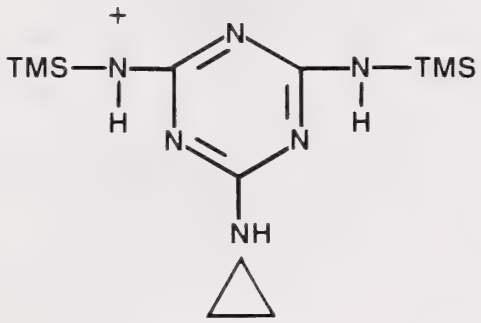
Refer to facing page.

II. CONFIRMATORY METHOD

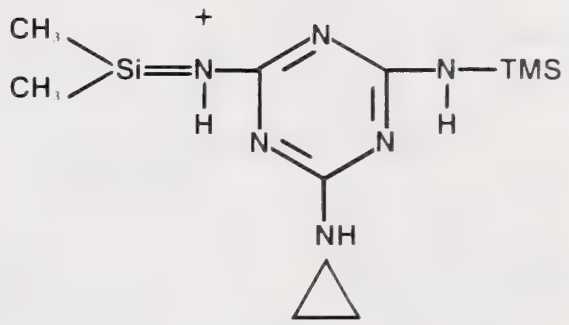
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Postulated ion fragments.

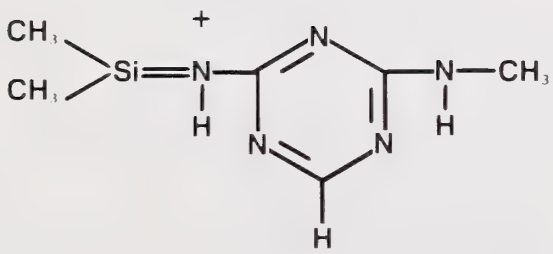
CYROMAZINE



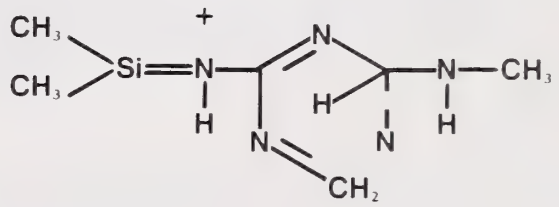
m/z = 310



m/z = 295

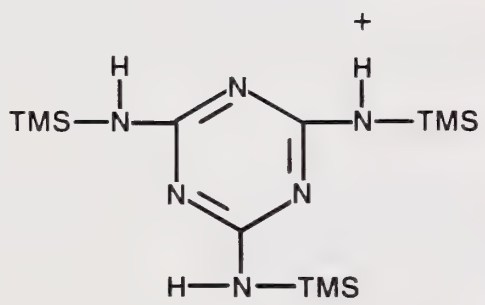


m/z = 181

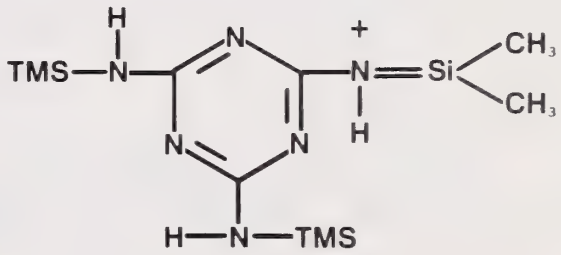


m/z = 171

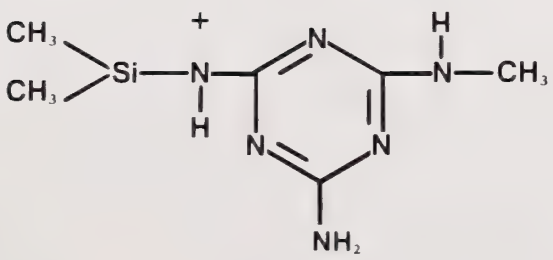
MELAMINE



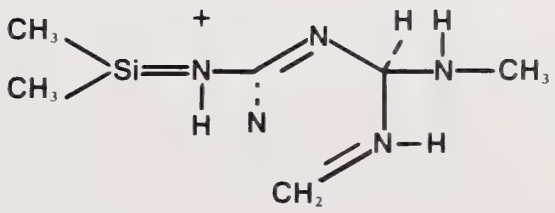
m/z = 342



m/z = 327



m/z = 197



m/z = 171

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Procedure Steps

Refer to Determinative Method, section H, in addition to the following:

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Acetone Methanol	Vapors are irritating to the skin, eyes, and respiratory system.	Perform all work in an efficient hood. Avoid contact with skin, eyes.
E. Derivatization		
BSTFA + 1% TCMS TCMS	Combustible liquid. It is also a suspected mutagen/carcinogen.	Same as above.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb for both compounds.

b. No false negatives at 50 ppb or higher in poultry or 100 ppb or higher in beef for both compounds.

2. Critical Control Points and Specifications

Refer to Determinative Method, section J.2, in addition to the following:

Record	*Acceptable Control*
a. Derivatization	110° C ± 2° C for 1 hr ± 0.1 hr
b. Sample cooling	−15° C to −30° C. The derivatives are stable for at least 20 hrs within the temperature range.
c. GC unit	Verify to ensure that the injector of the system is clean and free of contaminants by injecting 2 μL of BSTFA + 1% TMCS.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Analyze three 50 ppb (mixture of cyromazine and melamine) per day for a three-day period.

ii. Phase II: Fortified samples—Analyze three chicken samples and three beef samples at 100 ppb. Demonstrate that the coefficient of variation for the relative ion ratios is within 10% for each species. Also make sure the method blank is free of interferences. Repeat experiment for two more days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Check samples for analyst accreditation.

(a) Three samples for each species, including one blank. If fortified, the sample should be fortified at 50 ppb for poultry and 100 ppb for beef. The same check sample can be used in both the determinative and confirmatory methods.

(b) Report data to the Chemistry Division.

b. Acceptability criteria.

Refer to section F.3.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples	Refer to Determinative Method, section J.4.
5. Sample Acceptability and Stability	Refer to Determinative Method, section J.5.
6. Sample Set	Each set must contain a method blank, a blank tissue fortified at the level found in the presumptive suspect sample, and all positive samples found above the concentration of interest from the determinative method.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): 50 ppb (poultry), 100 ppb (beef).

MERCURY

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 5
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 9
G. Calculations 10
H. Hazard Analysis 11
I. [Reserved]
J. Quality Assurance Plan 12

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The sample is digested with a sulfuric acid-potassium permanganate solution to free all organic mercury compounds as ionic mercury. Excess permanganate is destroyed with hydroxylamine, and further reduction with stannous chloride to metallic mercury makes possible the measurement of the mercury vapor in the air. The lower limit of detection is 0.01 μg Hg.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Reaction flasks, pear-shaped, 100 mL, 14/20 standard taper (Kontes Glass Company, Vineland, NJ 08360, K-294250 or equivalent).

b. Continuous flow spectrophotometric cell, cylindrical, 100-150 mm, with tube on each end with push backs. (Optical Cell Company, Inc., 10792 Tucker Street, Beltsville, MD 20705—No. 4-435 or equivalent).

c. The aeration equipment is illustrated in Figure 1 on the facing page. If continuum background correction is unavailable, concentrated sulfuric acid can be put in the bottom of the water trap to within 5 mm of the end of the tube. Any number of compounds, such as hydrocarbons of water, display molecular absorption in this region.

d. Aeration tube should have an extra coarse frit (Kontes Glass Co., Sketch No. 001603-21, Quotation No. 37866, or equivalent).

e. Water trap (Kontes Glass Co., Sketch No. 001602-21, Quotation No. 37866, or equivalent).

f. Modified 3-way stopcock: Figure 2 on page MER-4 shows a full-sized view. (Kontes Glass Co., Quotation No. 38106, or equivalent.)

g. Flowmeter: Should have a range of 0-1.7 L/min—Brooks Sho-Rate with a R-2-15A tube using a steel ball (A. H. Thomas Company, P.O. Box 779, Philadelphia, PA 19105, 5545-B [case] and 5545 [tube]), or equivalent.

2. Instrumentation

Atomic absorption spectrophotometric equipment capable of continuum background correction is employed in the analysis. A mercury electodeless discharge lamp (EDL) is used as a resonance source. With the EDL, background correction is difficult to achieve; therefore, a trap of concentrated sulfuric acid is used. (Refer to section G.2.c.)

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

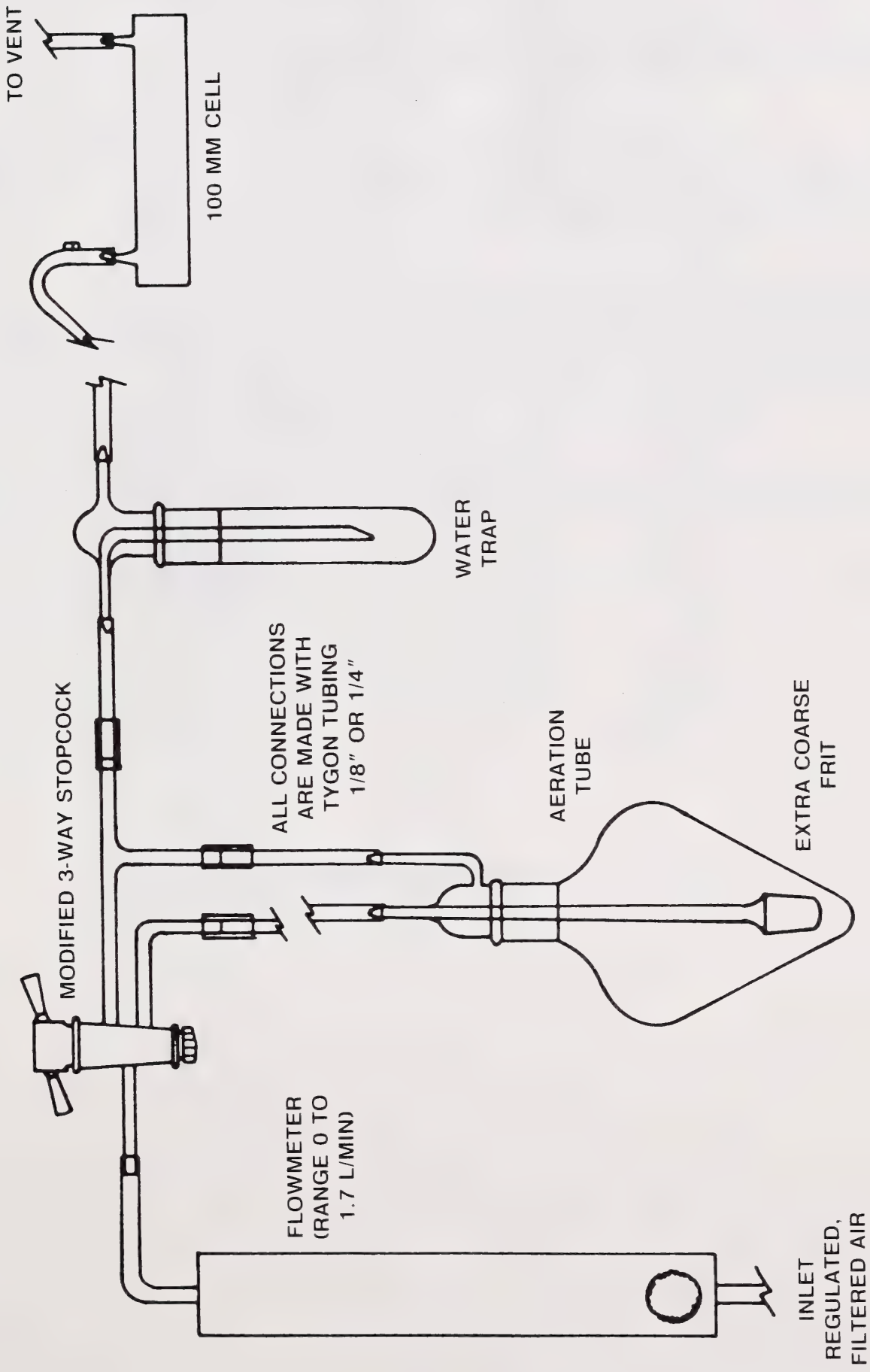


FIGURE 1—FLOW DIAGRAM FOR MERCURY ANALYSIS

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

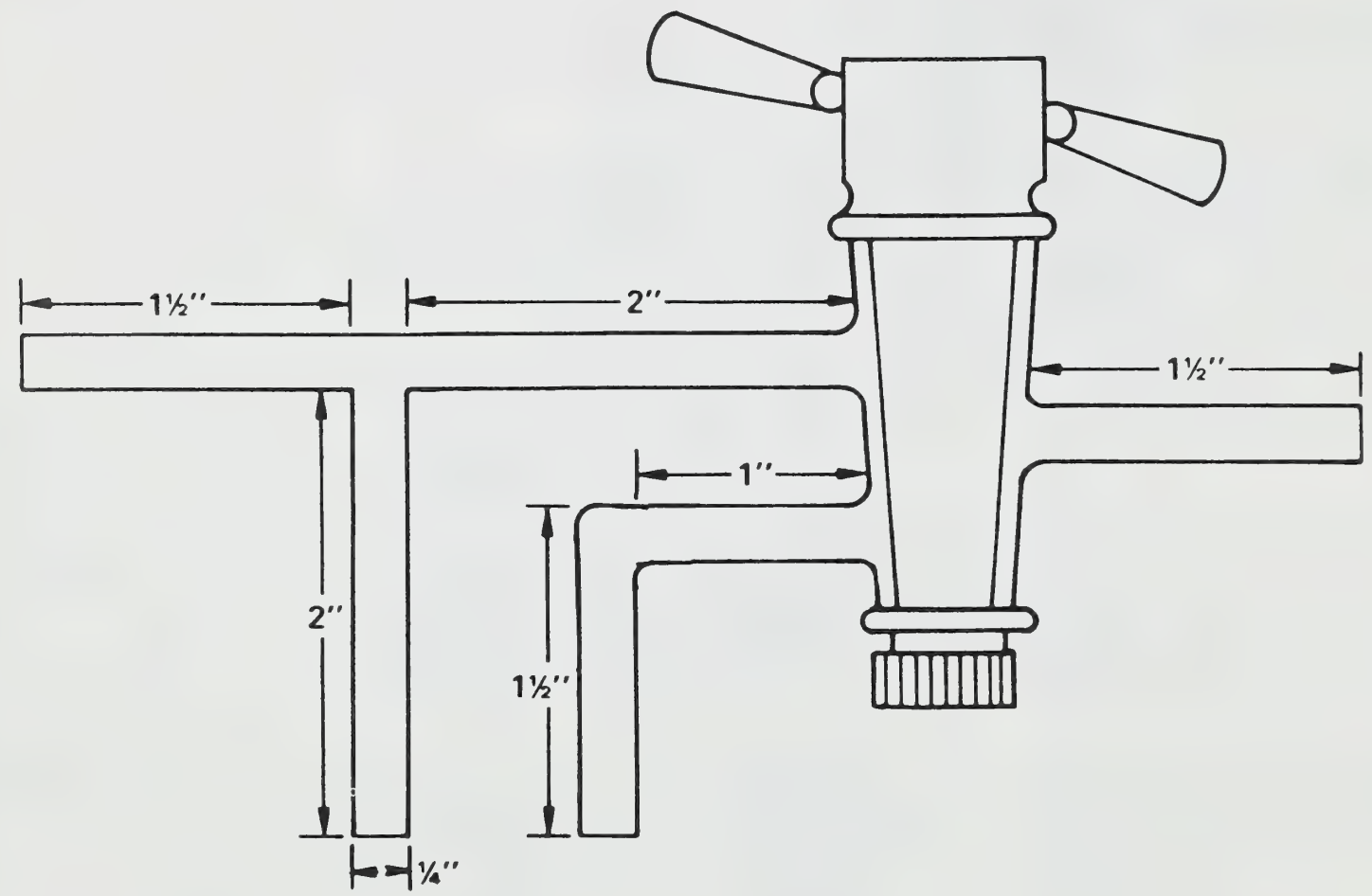


FIGURE 2—MODIFIED 3-WAY STOPCOCK

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solutions List

a. Concentrated reagent-grade nitric acid, 1 + 1 with water (v/v).

b. Hydroxylamine hydrochloride (NH_2 OH-HCl) solution, 10% (w/v): Dissolve 25 g of reagent-grade NH_2 OH-HCl in about 200 mL of distilled water. Transfer to a 250 mL volumetric flask, dilute to volume with distilled water, and mix well.

c. Potassium permanganate ($KMnO_4$) solution, 6% (w/v): Completely dissolve 60 g of reagent-grade $KMnO_4$ in about 800 mL of distilled water in a 1 L beaker using a heated magnetic stirrer. Transfer the solution quantitatively to a 1 L volumetric flask, cool, dilute to volume with distilled water, and mix well.

d. Stannous chloride ($SnCl_2$) solution, 10% (w/v). Prepare fresh every week. Dissolve 20 g of reagent-grade $SnCl_2 \cdot 2H_2O$ in 40 mL of warm concentrated hydrochloric acid. When all the stannous chloride has dissolved, add 160 mL of distilled water. Mix well and store in a 250 mL reagent bottle.

e. Sulfuric acid (H_2SO_4) concentrated: reagent grade.

NOTE: Before committing samples, check different suppliers and check each lot of compounds b through e. The amount of mercury found in them varies markedly.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Inorganic mercury standard, 1000 μg/mL (Fisher Scientific Co., 711 Forbes Ave., Pittsburg, PA 15219; catalog No. SO-M-114 or equivalent).

b. Organic mercury standard, 1000 μg/mL: (Alfa Products, P.O. Box 299, 152 Andorf Street, Danvers, MA 01923; catalog No. 88036, or equivalent).

2. Preparation of Standards

a. Mercury standard solution: 10 μg Hg/mL. (NOTE: 100 μg/mL standard may be prepared as an intermediate step if desired.) Pipet 1.0 mL of standard 1.a into a 100 mL volumetric flask containing 2 mL of redistilled nitric acid. Dilute to volume with distilled water and mix well.

b. Preparation of standard curve. Make fresh daily.

mL reagent	*final volume with Distilled water*	*μg Mercury in 20 μL*
1	10 mL	0.02
3	10 mL	0.06
5	10 mL	0.10

c. Methyl mercury standard solution: 10 μg/mL. Pipet 1.0 mL of standard 1.b into a 100 mL volumetric flask, add 2.0 mL redistilled concentrated nitric acid and dilute to volume with distilled water.

d. 5.0 μg/mL fortification standard. Pipet 5.0 mL of standard solution 2.c into a 10 mL volumetric flask and dilute to volume with distilled water.

NOTE: If samples are encountered with higher amount of Hg, the standard curve (refer to step 2.b) may be extended using 0.2, 0.6 and 1.0 μg Hg in 20 μL standard solutions.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Muscle tissue—Eliminate as much fat from tissue as possible. Pass rapidly three times through food chopper with plate opening less than or equal to 1/8 inch, mixing thoroughly after each grinding.

b. Liver or Kidney—Eliminate as much fat and connective tissue as possible from both the kidney and liver. Place tissues in a separate blender jar and blend unitl well homogenized (Waring Blender should be used with extreme care when blending). Blend 1 min; permit blender to cool for not less than 1 min before blending again. Do not use variable transformers to control speed of blender. Freeze tissues until determinations are ready to be run on each.

c. Hair—Wash with plain tap water to remove extraneous material, rinse with distilled water, and then dry. Place in freezer until ready to start determinations.

2. Sample Extraction

a. Clean all glassware with concentrated nitric acid (refer to section C, Reagent and Solution List, item a) and rinse with distilled water just before *each* use.

NOTE: Avoid use of towels, which may contain mercury, and do not use detergents.

b. Place approximately 0.600-0.750 of the homogenized tissue (muscle, liver, or kidney), or 0.5 g hair, into a tared 100 mL pear-shaped flask, taking care that all of the sample is deposited in the bottom of the flask and none is left in the neck. Reweigh the flask and obtain the sample weight to the nearest 0.01 g, by difference. Cap the flask with a clean 10 mL beaker inverted over the top of the flask. This beaker is left on the flask during all stages of the digestion procedure.

c. Pipet 5.0 mL of concentrated H_2SO_4 into the flask and place it on a steam bath to digest the sample (20-45 min is usually sufficient). Swirl flask during digestion to break up particles. The completed digested sample will form a highly colored solution with no pieces of undissolved matter, although the solution may be slightly cloudy.

NOTE: Great care must be exercised at this point that all the sample is actually in solution and that there are no particles on the sides of the flasks or suspended in the solution. If this is not done, the digestion will not be complete.

d. When the sample is digested, place the flask into an ice bath for 5-10 min. Then pipet 15.0 mL of 6% $KMnO_4$ solution into the flask and swirl the flask, gently at first and then vigorously, until sample is well mixed. Place sample in rack and continue until $KMnO_4$ has been added to all samples.

e. Swirl and place the flask on steam bath and allow the sample to digest further. Swirl the flask occasionally, and continue to heat until frothing ceases and all foam disappears (usually 15-20 min). Do not heat longer than is needed. Some foam may be present when reaction has stopped.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

f. Remove the flask from the steam bath and pipet a further 5.0 mL of 6% $KMnO_4$ (10.0 mL of 6% $KMnO_4$ for hair samples) solution into it.

g. Place the flask back on the steam bath for 15 min.

h. Cool the flask to room temperature and analyze the contents for mercury by atomic absorption.

3. Internal Standard for Checking Analytical Performance

Using a microliter syringe, add 20 μL (0.10μg) of fortification standard (refer to section D.2.d to 0.75 g of homogenized tissue in 100 mL pear-shaped flask. Proceed as in steps section E.2.a-h.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Preparation of a Calibration Curve

NOTE: To be carried out at least once per day.

a. Into each of eight clean 100 mL flasks, pipet 20 mL 6% $KMnO_4$ solution (refer to section C, Reagent and Solution List, item c). Cap the flasks with clean 10 mL beakers inverted over the tops of the flasks.

b. Cool the flasks in an ice bath for a few minutes, then slowly and cautiously pipet 5.0 mL of concentrated sulfuric acid into each flask. Swirl gently and allow to cool.

c. Using a microliter syringe, add 20 μl of each inorganic standard (1 μg, 3μg, and 5 μg/mL) so that duplicate standards are obtained for each level. The levels will be 0 (none added), 0.02 μg, 0.06 μg, and 0.10 μg.

d. Cool the flasks to room temperature prior to the aeration and atomic absorption analysis.

NOTE: Reagent blanks should show absorbances which are equivalent to 0.02 μg or less of Hg. If reagent blanks are higher, check glassware cleanliness and reagent solutions. Some checking of various suppliers' reagents may be necessary to determine those most suited to this analysis.

2. Atomic Absorption Analysis

a. Set up the atomic absorption equipment according to the diagram in Figure 1. Set the air flow to give good sensitivity and low foaming (0.7-1.0 L/min).

b. Add 5.0 mL of 10% $NH_2OH \bullet HCl$ solution (10.0 mL in the analysis of hair) to the digestion flask and swirl to dissolve the managanese oxides. Add about 10 mL of distilled water to bring the total volume to 40 mL $\pm$ 2.0 mL.

NOTE: This solution should not have any color or any particles suspended in it, but it may be slightly cloudy. See NOTE in section E.2.c.

c. Add 2 mL of 10% $SnCl_2 \bullet 2H_2O$ solution and immediately aerate the solution.

d. Discontinue the aeration after the recorder pen has settled back to within a few chart divisions (2 or 3) of its original baseline—usually 1 to 1½ min depending on the actual aeration rate.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

a. Calculate area of peak by measuring its height in mm and multiplying by the mm width at one-half its height (read to nearest 0.25 mm). Standard curve is calculated using linear regression.

The linear regression formula is: $y = mx + b$

Where: $y = \mu g/g$, x = area, m = slope, b = intercept

b. Calculate m and b. Calculate μg mercury in sample and divide by sample weight in grams to obtain ppm.

2. References

a. ''Determination of Mercury in Fish (Atomic Absorption Spectrophotometric Method),'' Method CAS-AM-70.10, June 11, 1970, Dow Chemical Company, Midland, MI 48640.

b. Manning, D. C. ''Compensation for Broad-Band Absorption Interference in the Flameless Atomic Absorption Determination of Mercury,'' *Atomic Absorption Newsletter*, Vol. 9, No. 5 (Sept-Oct 1970), pg. 109.

c. Kothandaraman, P., and Dallmeyer, J. F., ''Improved Desiccator for Mercury Cold Vapor Technique,'' *Atomic Absorption Newsletter*, Vol. 15, No. 5 (Sept-Oct 1976), pg. 120-121.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination of Mercury in Liver, Muscle, Kidney or Hair by Atomic Absorption Spectrophotometry.

2. Required Protective Equipment

Safety glasses, face shield, heat resistant gloves, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Conc. nitric acid Conc. sulfuric acid	Acid fumes and/or spattering can result in burns or irritation of skin, eyes, and respiratory system.	Work in efficient fume hood and wear protective equipment at all times.
E.2. Sample Extraction		
Clean all glassware with nitric acid	See above.	Exposure is greatly reduced by working in a hood with a distilled water supply and a sink.
Pipet 5.0 mL conc. H_2SO_4	See above.	Use repipettor or equivalent.
Pipet 15.0 of 6% $KMNO_4$	Acid spattering when mixed with this strong oxidizing agent.	Add the $KMNO_4$ slowly to the cold acid digest.
F.2. Atomic Absorption Analysis	Acid spattering and mercury vapors that may produce cumulative toxic effects.	The reaction train used to introduce mercury vapor to the A.A. is pressurized. Therefore, it is important to check for cracks in the glass components and to work behind some protective shield.

4. Disposal Procedures

Acid digest	See above.	May be diluted with water and flushed down a waste disposal sink.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Element	*Analytical Range (ppb)*	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
Mercury	0-?†	80-110‡ (inorganic)	≤ 10	≤ 15
		> 55 (organic)		

†Upper limit of range dependent upon applicable dilution volume.
‡CV ≤ 15% (running average of last 10).

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Background absorbance, date, signature (unless background is electronically removed).	
b.	Reagent, lot no., date checked, findings, signature.	Combined reagents should show response equivalent to or less than 0.01 μg Hg.
c.	Standard, lot no., source, date made, solvent, signature.	10 μg stds. should be made monthly; all others daily. Solvent for 10 μg/mL dilute HNO_3; solvent for all others distilled H_2O.
		Samples should be homogeneous and show very little fat content.
		Glassware should be very clean with no leftover liver or grease present. Samples above 0.05 ppm Hg should be rerun in duplicate to check for any contamination. Blank should be ≤ 0.01 μg Hg.
d.	Weight of sample (on worksheet).	Weigh 0.600 to 0.750 g of liver, muscle, and kidney, or 0.500 g hair in bottom of flask. Record wt. to nearest 0.001 g. If sample remains on side, discard and reweigh.
e.	Time placed on steam bath and time removed (treat all samples the same).	After first 15 min, swirl to help dissolve tissue and check for undisolved tissue.
f.	Time placed on steam bath and time removed.	Place all samples on and take all samples off at same time. Heat only until reaction stops: 15-20 min.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	Record	*Acceptable Control*
g.	Time placed on and time removed.	15 min maximum.
h.	% recovery, date, analyst.	Should range between 80 and 110% (inorganic Hg), > 55% (organic Hg) at 0.1 μg level.
i.	Record by strip chart recorder.	With given setting for our instrument, 0.1 μg std should give approximately 50-60% deflection.
j.	Yes or no (on worksheet).	Total digestion shows no visible dark material. Some fat may be present if a fatty sample (e.g., bologna) is being run.
k.	Coefficient of correlation.	0.998-1.000

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—4 levels, 3 replicates each.

(a) 0.00 ppm.

(b) 0.02 ppm.

(c) 0.06 ppm.

(d) 0.10 ppm.

ii. Phase II: Fortified samples—4 levels, 3 acceptable replicates at same nominal level as above over a minimum of 3 separate days.

NOTE: Phase I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples from FSIS Science Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency—initially, minimum of 1 per set, reduced to 1 per week per analyst. This sample is an internal check sample. It is a pooled sample analyzed at least 10 times to obtain a ''running'' average.

ii. A recovery (or recoveries) is analyzed with each set of samples. Also, a ''blank'' tissue is analyzed, and a running average is maintained for both the blank and recovery. Blank tissue matrix should not be from a single tissue source. Each species should be tested separately. Records are maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver, kidney, muscle, hair.

b. Sample receipt size: Varied; enough to obtain matrix for all required quantitative tests and reserve sample.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage:

i. Time: Indefinite.

ii. Condition: Frozen.

6. Sample Set

a. Reagent blank.

b. Tissue blank.

c. Fortified tissue at level of interest.

d. Sample(s).

7. Sensitivity

a. Lowest detectable level (LDL): 0.02 ppb.

b. Lowest reliable quantitation (LRQ): 0.01 ppb.

c. Minimum proficiency level (MPL): 0.02 ppb.

MELENGESTROL ACETATE

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 10
G. Calculations 13
H. Hazard Analysis 14
I. Worksheet 15
J. Quality Assurance Plan 19

II. Confirmatory Method

A. Introduction 22
B. Equipment 22
C. Reagents 22
D. Standards 22
E. Sample Preparation 22
F. Analytical Confirmation Procedure 23
G. [Reserved]
H. Hazard Analysis 25
I. Worksheet 25
J. Quality Assurance Plan 25

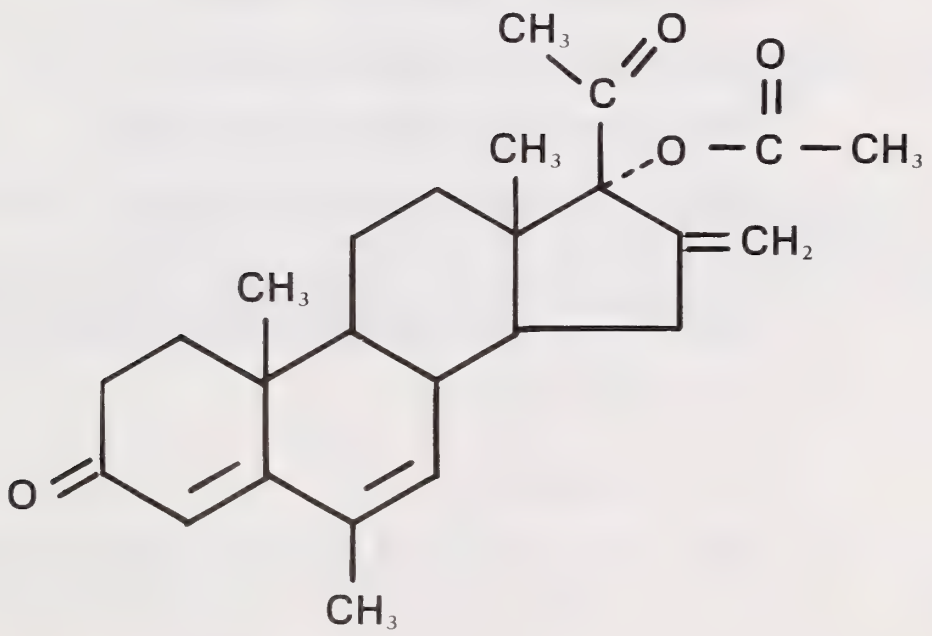
I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Melengestrol acetate (MGA) is a progestational agent added to the feed of heifers to suppress estrus and thereby achieve an increase in feed efficiency and the rate of weight gain. It is regulated as a suspect carcinogen in feedlot heifers.

It is extracted from bovine fatty tissue with hexane and partitioned into saturated acetonitrile. The acetonitrile mixed with hexane is evaporated to dryness. The residue is redissolved in hexane (while the flask is hot), placed on a Florisil column, washed with 95:5 hexane:acetone, and eluted with 75:25 hexane:acetone. The eluate is evaporated to dryness, redissolved in hexane (while the flask is hot), and injected onto a 4-foot column, packed with 3% XE-60 or 1% OV-17 on Gas Chrom Q, 80/100 mesh, in a gas chromatograph equipped with an electron capture detector.



2. Applicability

This method is applicable to the analysis of MGA in bovine fatty tissue.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Flask, boiling, round bottom, 500 mL.

b. Funnel, separatory, Teflon stopcock, 500 mL.

c. Funnel, 100 mL.

d. Rotary evaporator with control water bath (30-100° C).

e. Pipettes, volumetric (0.5, 1, 2, 5, and 10 mL).

f. Flask, volumetric (10, 25, 50, and 100 mL).

g. Reservoir—24/40 male joint in the bottom, 500 mL (Kontes K422450-9007).

h. Stirring rods.

i. Beakers, 250 mL.

j. Teflon stoppers, sizes 9, 16, and 27.

k. Water bath with temperature range 30-100° C.

l. Reacti-therm block heater—Pierce #18800.

m. Stopcock—Teflon, variable flow plug, #2 plug size 11/25 mm (Kontes #821111).

n. Chromatography columns 400 mm × 19 mm, fitted with medium porosity sintered glass disc, 24/40, Cat. No. K420550, Kontes size 224.

o. Glass syringe, 10 μL.

2. Instrumentation

a. Gas chromatograph with Ni^{63} electron capture detector.

b. Glass column for GC, 4 ft × 2 mm ID containing 3% XE-60 on Gas Chrom Q, 80/100 mesh, optimized for 0.75 ng MGA to yield 1/2 full scale deflection.

I. DETERMINATIVE METHOD

C. REAGENTS

Reagents List

a. Acetone, distilled in glass.

b. Hexane, distilled in glass.

c. Acetonitrile, distilled in glass.

d. Sodium sulfate, anhydrous granular.

e. Florisil, 60-100 mesh, certified to have been activated at 660-675° C, stored in glass bottle, and put in 130° C oven for 48 hrs before use. Fisher F-100 or equivalent.

f. Glass wool, silanized.

g. Solvent mixture (ratio by volume):

i. 95:5 (hexane 95 mL:acetone 5 mL)

ii. 80:20 (hexane 80 mL:acetone 20 mL)

iii. Saturated acetonitrile (acetonitrile saturated with hexane)

iv. 75:25 (hexane 75 mL:acetone 25 mL)

NOTE: All solvents must show no impurities when tested as follows: Evaporate to dryness 400 mL solvent, transfer to 10 mL volumetric flask, bring up to final volume with hexane, and inject 3 μL on the gas chromatograph. There should be no broad solvent front and no peaks at the retention time of MGA.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

The Upjohn Co. (melengestrol acetate, 99.5% pure).

2. Preparation of Standards

a. Stock solution I, 500 μg/mL

Quantitatively weigh 50 mg MGA standard and transfer to 100 mL volumetric flask with 80:20 hexane:acetone; dissolve the MGA and dilute to volume with the same solvent mixture.

b. Stock solution II, 25 μg/mL

Dilute 5 mL of stock solution I to 100 mL with hexane.

c. Working solution I, 0.125 μg/mL

Dilute 0.5 mL of stock solution II to 100 mL with hexane.

d. Working solution II, 0.25 μg/mL

Dilute 1.0 mL of stock solution II to 100 mL with hexane.

e. Working solution III, 0.375 μg/mL

Dilute 1.5 mL of stock solution II to 100 mL with hexane.

f. Recovery curve fortifications

i. Add 2.0 mL of working solution I to 25.0 g of blank tissue (equivalent to 10 ppb)

ii. Add 2.0 mL of working solution II to 25.0 g of blank tissue (equivalent to 20 ppb)

iii. Add 2.0 mL of working solution III to 25.0 g of blank tissue (equivalent to 30 ppb)

3. Storage Conditions

All standards should be stored in tight sealed glass bottles at 0-4° C.

4. Shelf Life Stability

a. Stock solution: one year.

b. Working solution: three months.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 25 g of tissue into a 250 mL beaker.

b. Weigh four 25 g of blank tissue. Use them as follows:

   i. 1 control blank.

   ii. 1 recovery standard. Fortify them at 20 ppb (per screening determination).

   iii. 3 recovery standards, as described in section D.2.f., for quantitation of positives from screening method.

c. Add about 22 g anhydrous sodium sulfate to the beaker with the tissue. (The sodium sulfate added to the sample in the beaker removes the moisture in the tissue and eliminates emulsions.)

d. Add 200 mL hexane:acetone (80:20).

e. Place sample in water bath at 60° C for 30 minutes or until the fat is melted.

f. Rinse a 100 mL funnel containing a small silanized glass wool plug and about 22 g anhydrous sodium sulfate (granular) with 10-15 mL acetone followed by 15-20 mL hexane, collecting rinse in the 500 mL separatory funnel. Remove the funnel, stopper the separatory funnel with the Teflon stopcock, and shake. Discard the rinse and allow the separatory funnel to drain dry. Replace the funnel and close the stopcock.

g. Filter the hexane-fat mixture through the funnel into the separatory funnel.

h. Wash the sides of the beaker with 20 mL of hexane and filter as in step g.

i. Add 150 mL hexane to the beaker and agitate with a stirring rod. Filter into the separatory funnel as in step g.

j. Add 50 mL acetonitrile saturated with hexane to the beaker and agitate and filter as in step g.

k. Rinse funnel with 12-20 mL hexane.

l. Remove funnel, stopper with the Teflon stopcock, and shake vigorously for 1 minute. Wait 15-20 minutes for the acetonitrile-hexane to separate.

m. Collect acetonitrile (bottom layer) in a previously rinsed 500 mL round bottom flask.

n. Add 50 mL acetonitrile saturated with hexane to the separatory funnel and shake as in step l.

o. Repeat step m.

p. Repeat step n.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

q. Repeat step m.

r. Repeat step n.

s. Repeat step m.

t. Discard the hexane layer.

u. Evaporate to dryness on a rotary evaporator; water temperature at 90° C.

v. Add 20 mL hexane to the hot round bottom flask and swirl.

w. Pack the chromatography column with 10-12 cm florisil and 5 g anhydrous sodium sulfate. Pack the column by tapping gently.

x. Wash the column with 100 mL hexane, 100 mL acetone, and 100 mL hexane. Let each wash come to the top of the sulfate bed before adding to the next wash.

y. Transfer the sample from the round bottom flask to the chromatography column, rinsing the flask with two 15-20 mL portions of hexane.

z. Place the reservoirs on the chromatography columns.

aa. Wash the chromatography column with 800 mL of 95:5 hexane:acetone.

bb. When the wash from step aa has reached the top of the sulfate bed, close the stopcock.

cc. Add 170 mL of 75:25 hexane:acetone to the chromatography column. Place a 500 mL round bottom flask under the column and collect the eluate at the rate of 2 drops/second. (This step is critical. The variable flow plug allows control of the rate at which MGA is eluted through the column for consistent recovery.)

dd. Evaporate the hexane:acetone to dryness on the rotary evaporator. Immediately add 10 mL hexane to the flask and swirl at 60° C.

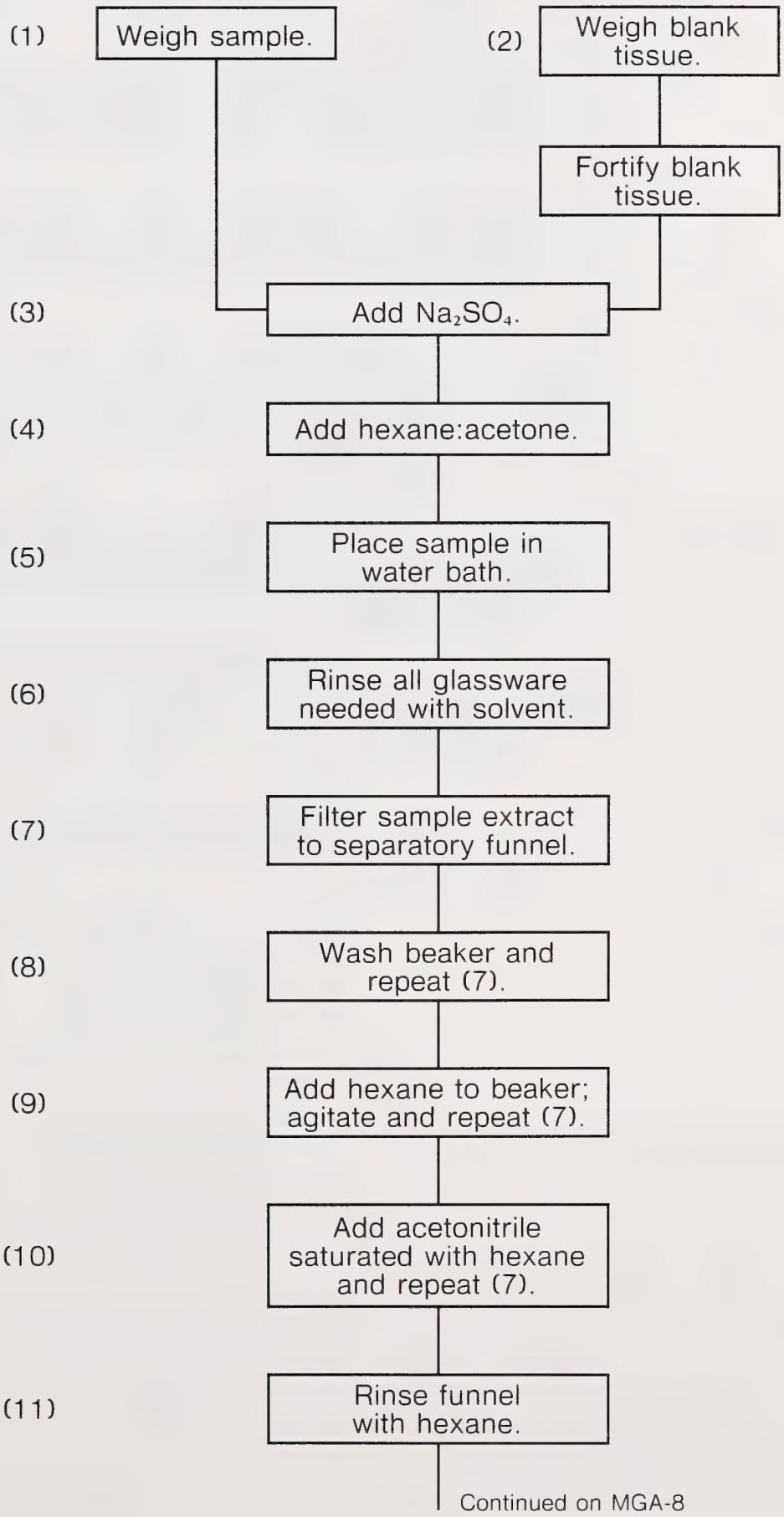
ee. Transfer the sample to a 15 mL test tube. Evaporate hexane under nitrogen to near dryness and adjust to 2.0 mL with hexane.

ff. The sample is ready for GC injection.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Flow Chart Summary



Continued on MGA-8

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

(12) Shake vigorously for 1 minute.

(13) Collect acetonitrile (bottom layer).

(14) Add acetonitrile saturated with hexane and shake.

(15) Evaporate to dryness (temp. at 90° C).

(16) Add 20 mL hexane; swirl.

(17) Pack florisil column.

(18) Wash column sequentially with hexane, acetone, and hexane.

(19) Transfer samples to column with 20 mL hexane.

(20) Rinse sample flask with two 20 mL portions of hexane and add to column.

Continued on MGA-9

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

(21) Wash columns with 800 mL of 95:5 hexane:acetone.

(22) Add 170 mL of 75:25 hexane:acetone.

(23) Evaporate to dryness using rotary evaporator. Add hexane to swirl.

(24) Transfer to test tube and evaporate using N-evap.

(25) Final volume to 2 mL with hexane.

(26) GC analysis.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

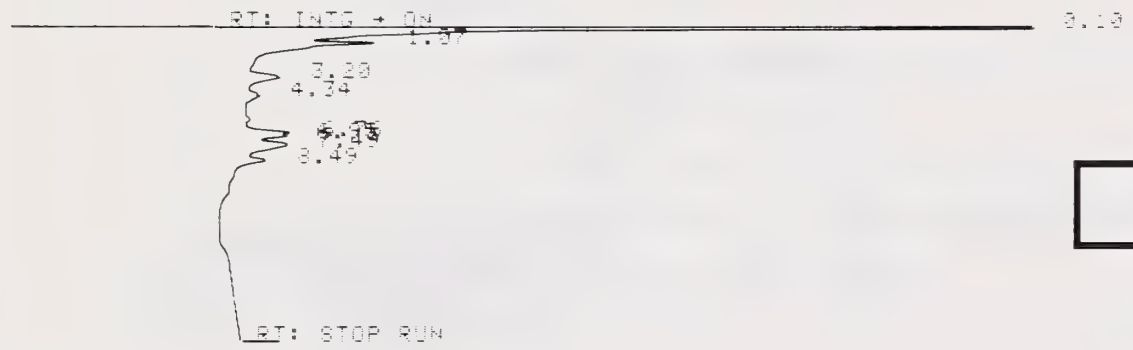
1. Instrumental Settings and Conditions

Oven temperature profile	
Initial	175° C (3 min)
Program rate	15° C/min
Final	240° C (20 min)
Detector temperature	350° C
Injection temperature	240° C
Injection mode	Set to inject 5 μL
Carrier gas	Argon/Methane
Flow rate	30-35 cc/min
Attenuation settings	2↑9
Volume injected	5 μL

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

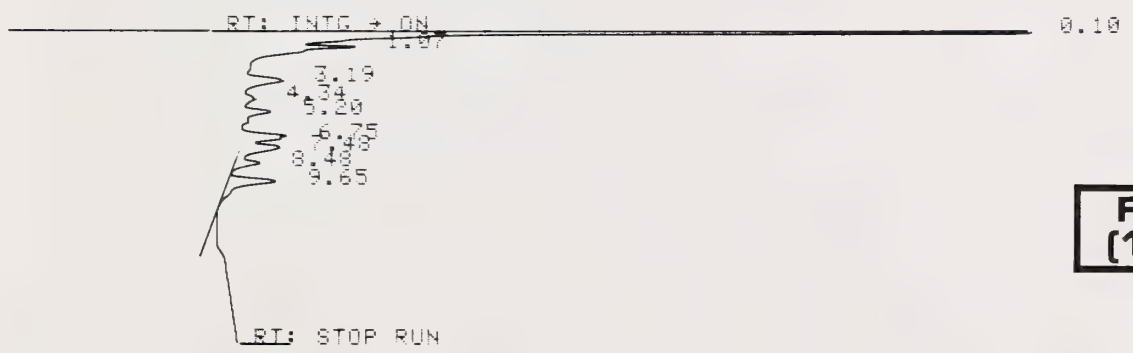
2. Sample Chromatograms



Control Blank (beef fat)

[hp] 5880A SAMPLER INJECTION @ 17:07 MAR 6, 1990
SAMPLE # : ID CODE :
10
AREA % COMPENSATED ANALYSIS

RT	AREA	TYPE	AREA %
0.10	434075.00	BB	94.389
1.07	5531.42	BB	1.203
3.20	7447.76	BB	1.619
4.34	3190.92	BB	0.694
6.95	714.19	BB	0.155
7.49	6896.10	BB	1.500
8.49	2025.50	BB	0.440



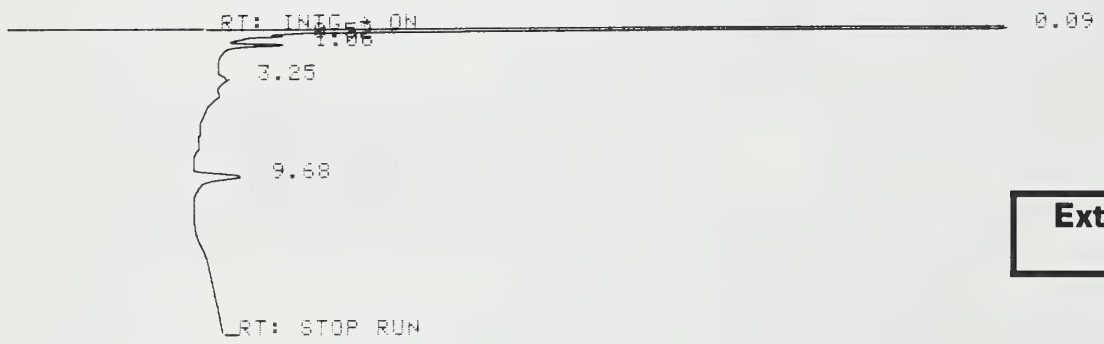
Fortified Standard (10 ppb) in beef fat

[hp] 5880A SAMPLER INJECTION @ 17:30 MAR 6, 1990
SAMPLE # : ID CODE :
11
AREA % COMPENSATED ANALYSIS

RT	AREA	TYPE	AREA %
0.10	348595.00	BB	88.733
1.07	6005.66	BB	1.529
3.19	8996.37	BB	2.290
4.34	2217.46	BB	0.564
5.20	4532.27	BB	1.154
7.48	6244.49	BB	1.590
8.48	3613.70	BB	0.920
9.65	12652.70	BB	3.221

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



External Standard (10 ppb)

[hp] 5880A SAMPLER INJECTION @ 17:54 MAR 6, 1990
SAMPLE # : ID CODE :
12
AREA % COMPENSATED ANALYSIS

RT	AREA	TYPE	AREA %
0.09	1076950.00	BB	98.094
0.52	75.35	BB	0.007
1.06	5258.68	BB	0.479
3.25	2508.92	BB	0.229
9.68	13081.20	BB	1.192

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

$$\text{ppb MGA} = \frac{(H/H')\ (C)\ (V/I)}{\text{g sample}}$$

Where:

H = Peak height of sample;
H′ = Peak height of standard;
C = ng MGA external standard injected;
V = total volume (mL) of sample solvent; and
I = mL sample solution injected.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Determination of Melengestrol Acetate Residue in Bovine Tissue

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Acetone Hexane Acetonitrile Isooctane	Flammable. Avoid breathing vapors. May cause skin irritation.	Keep in well closed containers in a cool place and away from fire. Use it under well ventilated hood.
Benzene	Highly flammable liquid. Exposure to vapors will cause irritation of mucous membranes, restlessness, convulsions, excitement, and depression. Benzene has been listed as a known carcinogen.	Same as above.

4. Disposal Procedures

Organic solvents	See above.	Dispose of in proper container.
Chromatography	See above.	Columns can be left under the hood until dry; the packing can then be disposed of in the trash can.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *MGA*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MGA

Analyst: _____ Matrix: _____

Date started: _____ Date completed: _____

Standard Curve Data

Standard	ppb	Peak Height
1		
2		
3		
4		

Linear Regression

b =
m =
r =
s =

Sample Identification	Liquid/Liquid Extraction Emulsion (y/n)	Column Elution 2 drops/sec (y/n)	Sample Weight	Peak Height	Amount Found (ppb)

Remarks:

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability %CV*
MGA	10-30	70-100	≤ 15

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	25.0 ± 0.1g
b. Sodium sulfate added to tissue	20-22 g
c. Water bath temperature	60° C
d. Liquid/Liquid extraction	No emulsion
e. Water bath temperature	90° C step E.1.u; 60° C step E.1.dd.
f. Addition of hexane	To hot flask
g. Column packing	Tight
h. Column elution	≤ 2 drops/second
i. Evaporation and hexane addition	All hexane/acetone evaporated and hexane added immediately

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards—Duplicate standard curve on each of 3 consecutive days, which will include the following:

(a) 0 ppb (0 μg/mL)

(b) 10 ppb (0.125 μg/mL)

(c) 20 ppb (0.25 μg/mL)

(d) 30 ppb (0.375 μg/mL)

ii. Phase II: Fortified samples—3 replicates at 0, 10, 20, and 30 ppb over a period of 3 different days using bovine fatty tissue.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Check samples for analyst accreditation.

(a) 15 bovine fat samples submitted by the supervisor or Laboratory QA Officer fortified at 0-30 ppb.

(b) Report analytical findings to Chemistry Division.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 every other week per analyst, or 20% of official samples analyzed.

ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All % recoveries.

(d) Running average, standard deviation, and CV for recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Bovine fat.

b. Sample receipt size, minimum: 50 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage:

i. Time: 6 months.

ii. Condition: −20° C.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set

a. Screening method.

For screening purposes, a sample set can include a tissue blank, a tissue blank fortified at 10 ppb, and up to 20 samples.

b. Quantitation method.

The sample set should include a tissue blank, a fortified standard curve including 0, 10, 20, and 30 ppb, and all samples found positive in the screening method.

7. Sensitivity

a. Lowest detectable level (LDL): 5 ppb.

b. Lowest reliable quantitation (LRQ): 10 ppb.

c. Minimum proficiency level (MPL): Not determined.

II. CONFIRMATORY METHOD

A. INTRODUCTION

Refer to Determinative Method, section A.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.

2. Instrumentation

HP 5980 GC/HP 5970 MS or equivalent, equipped with HP Ultra-performance 12 m × 0.2 mm i.d. fused silica methyl silicone film thickness 0.33 μm.

C. REAGENTS

Reagent List

a. Iso-octane—Burdick and Jackson, or equivalent.

b. HFAA—Heptafluorobutyric acid anhydride—Pierce #63164, or equivalent.

D. STANDARDS

Refer to Determinative Method, section D.

E. SAMPLE PREPARATION

Procedure

a. Dissolve the dry extract from Determinative Method, section E.1.ff, in 100 mL dry benzene.

b. Add 10 μL HFAA, cap, and mix by vortexing for 20 seconds.

c. Heat on a Reacti-Therm block for 1 hour at 70° C.

d. Remove, cool, and evaporate to dryness at room temperature under a stream of nitrogen.

e. Add 10 μL of 5% ethyl acetate in iso-octane and mix by vortexing for 20 seconds.

f. The sample is now ready for analysis.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

Instrument conditions:

a. Injector temperature	260° C
b. Interface temperature	300° C
c. Oven temperature program	
Initial temperature	40° C
Program rate 1	30° C/min to 150° C
Program rate 2	6° C/min to 300° C
Hold time	10 minutes
d. Ion dwell	100 milliseconds
e. Multiplier	800 relative to autotune

2. Required Samples

a. Inject 3 μL of reagent blank.

b. Inject 3 μL of external standard to check instrument performance.

c. Inject 3 μL of process blank.

d. Inject 3 μL of fortified standard and evaluate relative response to reference standard.

e. Inject 3 μL of sample.

3. Criteria for Confirmation

a. Ions monitored: 367, 381, 489, 517, 533, and 592.

b. Successful confirmation requires:

i. All six ions must be present.

ii. Molecular ion, 592, must be present at $\geq$ 3 times background noise.

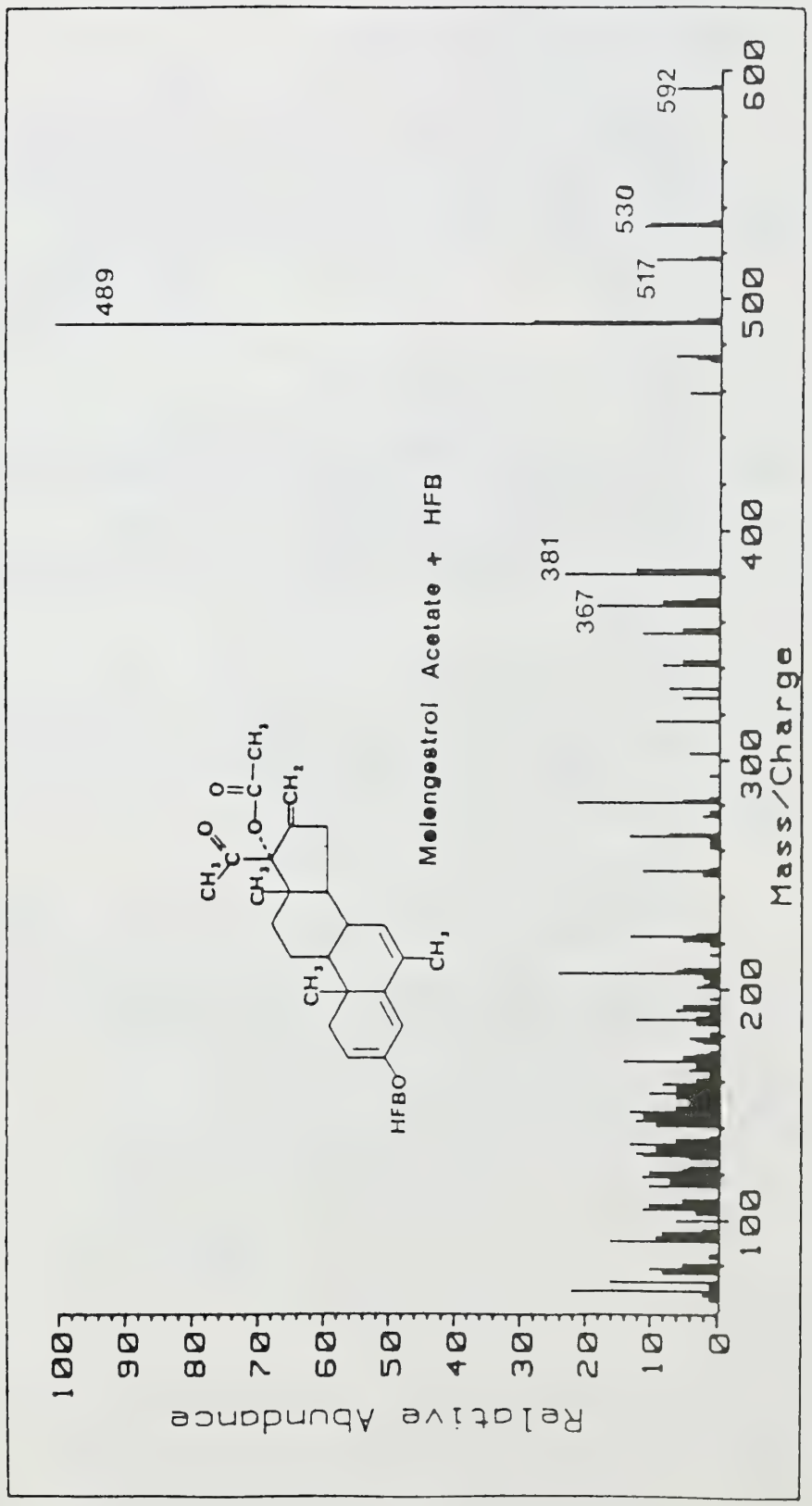
iii. Ion ratios (5) match within 15% of ratio found for the reference compound.

4. Interferences

None have been observed.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)



Full-scan electron ionization mass spectrum of MGA-HFB (40 ng)

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

Refer to Determinative Method, section H.

I. WORKSHEET

The worksheet on MGA-27, *Mass Spectrometry Data Report*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb.

b. No false negatives at 5 ppb.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Volume of benzene added	100 ± 10 μL
b. Volume of HFAA	10 ± 0.5 μL
c. Heating reaction	70° ± 5° C
d. Volume of ethyl acetate added	10 ± 0.5 μL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of the reference standard, determine the relative ratios of the ions of interest to show that the instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives fortified at 10 ppb.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: 3 check samples for analyst's qualification meeting the same requirement as Phase II.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division.

Notification from Chemistry Division is required to commence official analysis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	b. Acceptability criteria. Refer to section F.3.
4. Intralaboratory Check Samples	Refer to Determinative Method, section J.4.
5. Sample Acceptability and Stability	Refer to Determinative Method, section J.5.
6. Sample Set	Each set must include a blank tissue, a blank tissue fortified at the level of interest, and all samples found positive from the quantitation method.
7. Sensitivity	a. Lowest detectable level (LDL): 5 ppb. b. Lowest reliable confirmation (LRC): 10 ppb.

MASS SPECTROMETRY DATA REPORT

Internal Lab # _____ Form # _____ Date _____

Product/Tissue Type:

Analytical Request:

Analysis Performed:

Sample Wt. _____ Final Volume _____ Amount Injected _____

Findings:

Signatures: _____ Date: _____

_____ _____

INSTRUMENT CONDITIONS:

Gas chromatograph:
- Injection port—
- Column type—
- Initial temp—
- Final temp—
- Initial hold—
- Final hold—
- Program rate—
- Flow rate—
- Carrier gas—

Liquid chromatograph:
- Column type—
- Column size—
- Mobile phase—
- Flow rate/pressure—

Mass spectrometer:
- Transfer line temp—
- Source temp—
- Mode of operation—
- Calibration standard—
- Filament voltage—
- Filament current—
- Source pressure—
- Reagent gas—
- Vaporizer temp—
- Jet temp—
- Collision gas—
- Collision gas pressure—

MONENSIN IN TISSUES

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 11
G. [Reserved]
H. [Reserved]
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Monensin is extracted from experimental samples with methanol. Tissue extracts are processed by carbon tetrachloride or by silica gel column chromatography. Aliquots of the semipurified extracts in methanol are subjected to thin-layer chromatography on silica gel plates, and the antibiotic is detected on the TLC plates by bioautographic techniques using *Bacillus subtilis* as the assay organism. Monensin levels are estimated by comparing zones of inhibition from experimental samples to those from standard recoveries. Samples are authenticated by Polaroid photography.

2. Applicability

This method is applicable to chicken fat only.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

a. Tissue blender: Hamilton Beach Model 8, or equivalent, equipped with blender heads to fit half-pint Ball Mason jars.

b. Centrifuge: Servall Model NSE, or equivalent.

c. Rotary vacuum evaporator: Rinco evaporator, or equivalent.

d. Thin-layer chromatographic developing chambers: Brinkmann Instruments, Inc., Westbury, NY, or equivalent.

e. Photographic equipment: Polaroid Model MP 3, or equivalent.

f. Incubator maintained at 37° C.

g. Colorimeter: Spectronic 20, or equivalent.

h. Water bath.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane A.R.

b. Methanol A.R.

c. Chloroform A.R.

d. Benzene A.R.

e. Carbon tetrachloride A.R.

f. Ethylene glycol monomethyl ether A.R.

g. Chlorofrom A.R., containing 5% by volume of methanol A.R.

h. Silica gel G for thin-layer chromatography, Brinkmann Instruments, Inc., Westbury, NY, or equivalent.

i. Silica gel 0.2 to 0.5 mm for column chromatography, Brinkmann Instruments, Inc., or equivalent.

j. Glass wool.

k. Sodium sulfate anhydrous A.R. (granular).

l. Agar medium: Dissolve 0.69 g $K_2HPO_4 \cdot 3H_2O$, 0.45 g KH_2PO_4, 2.5 g yeast extract (Difco), 10 g glucose (cerelose), and 6.0 agar in enough deionized water to give 1 L total volume. Autoclave the solution for 20 min at 121° C.

   i. Ion agar, 2S (Colab Laboratories), or equivalent.

   ii. Noble agar (Difco), or equivalent.

   iii. Agar granulated, No. 11849 (BBL), or equivalent. Use at 1% concentration.

m. The following two commercially prepared plates or their equivalent can be used.

   i. Q47G silica gel TLC plates, Quantum Industries, 341 Kaplan Drive, Fairfield, NJ.

   ii. Woelm silica gel G TLC plates, Analtech, Inc., 75 Blue Hen Drive, Newark, DE.

   iii. Laboratory-prepared thin-layer chromatography plates can be used. Prepare a slurry of 26 g silica gel G in 55 mL of deionized water and apply to standard 20 × 20 cm glass plates at a thickness of 250 microns. Air-dry the plates for 10-15 min. Activate the plates in a drying oven by heating at 50° C for 15 min, 90° C for 15 min, and finally 110° C for 30 min. Following activation, allow the plates to cool slowly to room temperature.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

n. *Bacillus subtilis* stock solution.

i. Wash the growth of *Bacillus subtilis*, American Type Culture Collection, No. 6633, from one penassay seed agar slant (culture medium No. 1, Grove and Randall, Assay Methods of Antibiotics) with 3-5 mL sterile distilled water onto seeded agar with 0.03% manganous sulfate added.

ii. Incubate the culture for one week at 37° C.

iii. Following incubation, wash the growth from the agar surface of the Roux bottle with approximately 50 mL of sterile distilled water.

iv. Transfer the wash to a sterile 250 mL centrifuge bottle and hold the organisms at 65° C in a water bath for 30 min.

v. Centrifuge the suspension and discard the supernatant liquid.

vi. Repeat the resuspension and washing of the organisms three times.

vii. After the final wash, heat-shock the cells again by immersion in the 65° C water bath for 30 min and suspend them in 30 mL sterile deionized water.

NOTE:This stock suspension is stored at 4°-5° C for use.

o. *Bacillus subtilis* inoculum: Prepare the inoculum fresh weekly by diluting the stock suspension with sterile distilled water to obtain a 20% light transmittance at 530 nm using a Spectronic-20 colorimeter.

p. A 2 mg/mL solution of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) in 10% methanol:distilled water.

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards	Standard solutions. a. Weigh a quantity of standard sodium salt of monensin to contain 50 mg of monensin activity. b. Transfer the standard quantitatively to a 50 mL volumetric flask and dissolve the salt in methanol. Dilute to the mark with methanol and mix thoroughly. This standard solution contains 1000 μg/mL of monensin activity. c. Pipette 1 μL of 1000 μg/mL standard into a 200 mL volumetric flask with a volumetric pipette. Dilute to the mark with methanol and mix thoroughly. This standard contains 5 μg/mL of monensin activity.
2. Storage Conditions	Stock solutions should be stored tightly stoppered at 4° C.
3. Shelf Life Stability	Standard solutions are stable at least 10 days when stored under refrigeration as described in section 3.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Lean, Liver, and Kidney Tissue Extraction and Purification

a. Weigh a representative ground or minced tissue sample (approximately 30 g) into a half-pint extraction jar.

b. Add 2 mL methanol per gram of tissue.

c. Blend sample until uniform.

d. Transfer sample to centrifuge tubes and centrifuge at – 10° for 10-15 min (maximum rating of Servall Model NSE, 7500 rpm). (A refrigerated centrifuge is recommended to aid in removal of lipids.)

e. Transfer a measured volume of supernate (ca 1.5 mL/g tissue) to a 250 mL separatory funnel.

NOTE: In a series of samples, calculation may be expedited by using a uniform aliquot size based on the maximum amount of supernate obtained from the smallest sample.

f. Extract the supernate with three 30 mL portions of carbon tetrachloride. Combine the CCl_4 fractions in a 300 mL evaporating flask and evaporate to dryness by rotary vacuum evaporation, using a water bath at 50°-60° C.

NOTE: Approximately 2 min is required to complete the carbon tetrachloride extraction on each sample. Shake the MeOH extract and CCl_4 thoroughly and allow complete separation to occur before removing the CCl_4 fractions.

g. Transfer the sample with 3 mL of hexane to a 15 mL glass-stoppered sample tube. Rinse the flask with an additional 2 mL of hexane and transfer the rinse to the sample tube.

NOTE: Transfer can be made by pipette or by pouring carefully from the evaporating flask.

h. Evaporate the hexane using an N-Evap at 45° C under nitrogen.

i. Dissolve the sample in methanol using 0.05 mL methanol per 10 mL of supernate extracted.

2. Fat Tissue Extraction and Purification

a. Weigh a ground fat sample of approximately 20 g into a half-pint extraction jar.

b. Add 5 mL methanol per gram of fat and blend the sample until uniform.

c. Centrifuge sample for 10-15 min.

d. Transfer the supernate to a 300 mL evaporating flask and evaporate to dryness *in vacuo* (rotary vacuum evaporator) at 50-60° C.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

e. Prepare silica gel column for chromatography.

i. Place ca 10 mL hexane into a 14 × 250 mm (250 mL reservoir) glass chromatographic column. Insert a glass wool pledget and tamp with a glass stirring rod to eliminate air bubbles.

ii. Add 2.4 g (5 mL measured with a 10 mL graduated cylinder) of 0.2-0.5 mm silica gel to the column through a powder funnel and follow with ca 5 mL hexane.

iii. Stir the silica gel with a glass rod to eliminate air bubbles; then let the column stand until the silica gel settles. Drain a small volume of hexane through the column to facilitate packing.

iv. Add ca 2 cm of anhydrous sodium sulfate to the column, layering it carefully to avoid disturbance of the silica gel surface.

v. Drain the hexane to the top of the sodium sulfate.

vi. Prepare a separate column for each sample.

f. Dissolve the sample from step d in 10 mL hexane and charge the silica gel column with this solution at a flow rate of ca 3 mL/min. Rinse the evaporating flask with an additional 10 mL hexane and transfer the rinse to the column. Discard the hexane effluent.

g. Develop the silica gel column with approximately 100 mL chloroform at a flow rate of ca 3 mL/min. Discard the chloroform effluent.

h. Place an evaporating flask in position to receive the column effluent.

i. Elute the monensin from the column with 30 mL of chloroform containing 5% methanol.

j. Evaporate the eluate to dryness and transfer the sample to sample bottles as described in steps 1.g and 1.h.

k. Dissolve the sample in 0.1 mL methanol per 10 g of fat in the original sample.

3. Liver Tissue Extraction and Purification—Alternate Determination

This procedure is recommended if fat from liver samples interferes with the thin-layer chromatographic separation of monensin in steps 1.a-1.i.

a. Weigh a representative ground or minced tissue sample (approximately 30 g) into a half-pint extraction jar.

b. Add 2 mL methanol per gram of tissue.

c. Blend sample until uniform.

d. Transfer sample to centrifuge tubes and centrifuge at room temperature for 10-15 min (maximum rating of Servall Model NSE, 7500 rpm).

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

e. Transfer a measured volume of supernate (ca 1.5 mL/g tissue) to a 250 mL separatory funnel.

NOTE: In a series of samples, calculations may be expedited by using a uniform aliquot size based on the maximum amount of supernate obtained from the smallest sample.

f. Extract the supernate with three 30 mL portions of carbon tetrachloride. Combine the CCl_4 fractions in a 300 mL evaporation flask and evaporate to dryness by rotary vacuum evaporator using a water bath at 50°-60° C.

NOTE: Approximately 2 min is required to complete the carbon tetrachloride extraction on each sample. Shake the MeOH extract and CCl_4 thoroughly and allow complete separation to occur before removing the CCl_4 fractions.

g. Prepare silica gel column for chromatography as described for fat samples in step 2.e.

h. Dissolve the sample from step f in 10 mL hexane and charge the silica gel column with this solution at a flow rate of ca 3 mL/min. Rinse the evaporating flask with an additional 10 mL hexane and transfer the rinse to the column. Discard the hexane effluent.

i. Develop the silica gel column with approximately 100 mL chlorofrom at a flow rate of ca 3 mL/min. Discard the chloroform effluent.

j. Place an evaporating flask in position to receive the column effluent.

k. Elute the monensin from the column with 30 mL of chloroform containing 5% methanol.

l. Evaporate the eluate to dryness and transfer the sample to sample bottles as described in steps 1.g and 1.h.

m. Dissolve the sample in methanol using 0.05 mL methanol per 10 mL of supernate extracted.

4. Preparation of Standard Recoveries

Prepare negative control samples and standard recoveries for assay with each set of experimental samples.

NOTE: Prior to assaying samples, determine the linear range in recovery responses by testing several recovery samples. These samples should include those concentrations specified below in step b.

a. Weigh appropriate control tissue into half-pint blender jars.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Add standard monensin (5 μg/mL in methanol) by pipette to give the desired concentration in the recovery samples.

NOTE: One negative control (no monensin added), one recovery sample containing 0.04 μg/g wet tissue (0.04 ppm), one recovery sample containing 0.05 μg/g (0.05 ppm), and one recovery sample containing 0.06 μg/g (0.06 ppm) are generally processed with each set of experimental samples. However, additional recovery levels may be included if desired.

Control and recovery samples are extracted and chromatogaphed exactly as described for the corresponding tissue.

5. Thin-Layer Chromatography

Chromatograph the extracts from sections 1-4 above on thin-layer plates as follows:

a. Apply 20 μL of methanol extract of negative control sample, standard recovery samples, and experimental samples to a silica gel thin-layer plate. A forced-air hair dryer may be used to hasten evaporation of solvent during sample application.

NOTE: Eight or nine samples may be assayed on one thin-layer plate.

b. Apply negative control and recovery samples to each thin-layer plate in order to ensure adequate standardization of the procedure.

c. Label the TLC plate and draw a line in the silica gel with a pencil or a scribe 3 cm from the top of the plate to mark the solvent front.

d. Line a TLC developing chamber with a sheet of filter paper to improve saturation of the atmosphere in the chamber.

e. Prepare the developing solvent by mixing 80 parts carbon tetrachloride, 10 parts benzene, and 6 parts ethylene glycol monomethyl ether. Prepare solvent system daily and allow to equilibrate prior to plate development.

f. Pour the solvent down the sides of the developing chamber in order to saturate the filter paper while charging the chamber.

g. Develop the thin-layer plate in the chamber, allowing the solvent front to move up to the line previously scored on the plate.

NOTE: Development time is about 75 min.

h. Remove the plate from the chamber and allow the plate to air-dry on the laboratory bench. All traces of solvent should be removed from the plate.

6. Preparation of Bioautographs

Bioautographs are prepared according to the method of Kline and Golab, *J. Chromatog.*, 18, 409 (1965).

a. Melt agar medium (refer to section C, Reagent and Solution List, item I) in a steam bath.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Pour approximately 50 mL of melted agar into a 250 mL Erlenmeyer flask and fix the TLC plate by spraying the surface with the agar, using an atomizer-type sprayer attached to the laboratory compressed air supply.

NOTE: An artist's paint sprayer (Grumbacher atomizer #836, or equivalent) is satisfactory. Only a thin layer of agar should be sprayed on the TLC plate.

c. Place the TLC plate in a plexiglass holder (or other suitable device that will prevent contamination of the plate) and allow the plate to come to room temperature.

NOTE: This step is necessary to prevent too rapid solidification of the seeded agar overlay.

d. Cool 50 mL of melted agar medium to 54° C ± 2° C in a 125 mL Erlenmeyer flask.

NOTE: Cool slowly to prevent solidification of the agar before it can be inoculated and poured.

e. Inoculate the agar with 0.05 to 0.1 mL of *B. subtilis* inoculum (refer to section C, Reagent and Solution List, item o). Mix quickly by swirling.

NOTE: The inoculum may be adjusted to give adequate test sensitivity.

f. Pour this seeded agar over the surface of the TLC plate.

NOTE: Pouring of the seed agar must be done rapidly and carefully to ensure an even agar overlay before the agar solidifies. The plate may be slightly warmed to 37° C (but in no event to exceed 58° C) to facilitate equal agar distribution over the plate surface.

g. Allow the plate to cool until the agar sets.

h. Cover the TLC plate holder to prevent contamination and to prevent evaporation of moisture from the plate.

i. Incubate the bioautograph overnight (16-18 hrs) at 37° C.

j. Subsequent to incubation, spray the plate, using a reagent sprayer with a solution of INT (refer to section C, Reagent and Solution List, item p). Allow the color to develop for a period of approximately 2-4 hours. Additional spraying may be used to increase the rate and degree of color contrast.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Estimation of Concentration

a. After incubation, locate the monensin zones on the bioautograph at the R_f of 2.5 to 3.

b. Estimate monensin concentration by visually comparing zone sizes from experimental samples to zone sizes from standard recovery samples.

   i. Negative samples are reported as no activity at a sensitivity of the lowest possible standard recovery.

   ii. Positive samples are reported as the concentration of the corresponding standard recovery level or as a range between two standard recoveries.

   NOTE: Experimental samples yielding zones larger than the highest standard recovery should be diluted to yield zones within the range of the recoveries. Monensin levels obtained in this manner are regarded as approximate rather than absolute values.

c. If permanent records of bioautographs are desired, label the plates for identification and photograph by either transmitted or reflected light.

   NOTE: Polaroid photography of bioautographs has the advantage of producing a print immediately so that a satisfactory print may be obtained before destruction of the bioautograph.

d. Attempts to quantitate residues should not be made from photographs. Estimations should be made directly from the TLC bioautographic plate.

e. Test sensitivity when standard monensin is applied to TLC plates in methanol solution is 0.025 to 0.05 μg.

f. Test sensitivity defined by the standard recoveries processed by the extraction procedures described is generally 0.025-0.05 μg (0.025-0.05 ppm) monensin per gram of fresh tissue. However, test sensitivity is defined by the responses obtained on each individual TLC plate and the concentrations of the recoveries used with each set of test samples.

g. The assay must be regarded as semiquantitative and is more applicable to verifying the absence of monensin than to exact measurement of monensin levels. However, differences in zone sizes between standard recoveries can be noted and ranges in monensin concentrations can be estimated.

2. Reference

Eli Lilly, NADA 38-878V.

MORANTEL AND PYRANTEL DRUG-RELATED METABOLITES

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 8
F. Analytical Quantitation 10
G. Calculations 11
H. [Reserved]
I. [Reserved]
J. Quality Assurance Plan 12

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This assay is designed to detect and estimate morantel ((E)-1, 4, 5, 6-tetrahydro-1-methyl-2-[2-(3)methyl-2-thienyl)vinyl]pyrimidine and drug-related metabolites that are hydrolyzable to N-methyl-1, 3-propanedeiamine in bovine liver and muscle. Calibration curves are constructed to determine morantel residues in the range of 0.2 to 0.8 ppm in muscle and 0.4 to 1.2 in liver. It is also designed to detect and estimate pyrantel (trans-1, 4, 5, 6-tetrahydro-1-methyl-2-[2-(2-thienyl)vinyl]pyrimidine) and drug-related metabolites that are hydrolyzable to N-methyl-1, 3-propanediamine in swine liver and muscle. A calibration curve is constructed to determine pyrantel residues in the range of 0.4 to 1.2 ppm in muscle and 6 to 12 ppm in liver. Tissue containing morantel and metabolites or tissue containing pyrantel and metabolites are hydrolyzed in aqueous potassium hydroxide to yield N-methyl-1, 3-propanediamine, a product common to drug-related residues. N-ethyl, 3-propanediamine dihydrochloride is added as an internal standard to the sample before the hydrolysis step at a concentration equivalent to 0.4 ppm morantel or 4 ppm pyrantel in tissue. Following hydrolysis, both diamines are extracted into toluene, back-extracted into dilute aqueous acid, and converted to their *bis*-2-nitro-4-trifluoromethylphenyl derivatives with 4-fluoro-3-nitrobenzotrifluoride in a borax-buffered solution. The diamine derivatives are separated from the reaction mixture by extraction into n-hexane and purified by thin-layer chromatography prior to evaluation by pulsed electron capture gas-liquid chromatography.

Accurate results are obtained by referring the relative peak heights of the diamine derivatives of the pyrantel-related compound and the internal standard to a standard curve. This curve is constructed by relating, in a linear manner, the relative peak height response of calibration solutions of the diamine derivatives to a weight ratio of each. The concentrations of the standards are selected to optimize the working range of the GLC and to reflect residue levels ranging from one-half to two times the action level. A broader dynamic range may be employed, but the recommended range simplifies GLC calibration and assay of tissue extracts. From the established relationship of the relative response of the two diamines to weight ratio, and knowing the amount of internal standard added to the samples, the unknown concentration of morantel or pyrantel can be obtained by a graphical extrapolation or a linear regression calculation.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Precoated thin-layer plates: 20 × 20 cm, 250 μ thickness, LK6DF (channeled plate, preadsorbant area, silica gel, fluorescent indicator).

b. Desega multiplate developing tank: for ten 5 × 20 cm TLC plates, distributed by Brinkmann Instruments, Inc., Westbury, NY, or equivalent.

c. Centrifuge tubes, heavy-duty: 50 mL graduated (60 mL capacity), equipped with glass stoppers, Lab Glass, Inc., or equivalent.

d. Centrifuge tubes: 15 and 50 mL graduated, equipped with glass stoppers.

e. Volumetric flasks: 50, 100, 200, and 250 mL capacity, glass-stoppered.

f. Glassine paper.

g. Pipets, automatic transfer: 25 mL delivery volume.

h. Pipets, measuring: 0.5 mL delivery volume.

i. Pipet: ultramicro, 0.100 mL capacity, Scientific Glass Apparatus Co., Inc., JM-6840, or Medical Laboratory Automation, Mt. Vernon, NY.

j. Aluminum foil.

k. Electronic calculator equipped with a linear regression program.

l. Pipets, volumetric: 1, 2, 3, 4, and 5 mL delivery volume.

m. Pipets, serological: 10 mL delivery volume.

n. Pipets, Pasteur disposable.

o. Propipet bulb.

p. Adams pipet suction apparatus (No. C-5940), SGA Scientific, Inc., or equivalent.

q. Hamilton syringe: 10 mL capacity.

r. Screw-cap test tubes with Teflon liners, A. H. Thomas, #9447-E50, or equivalent.

s. Crystallizing dish: 190 mm (diameter) × 100 mm (height), for oil bath.

t. Scalpel.

u. Test tube rack.

v. Test tube mixer: Vortex mixer, or equivalent.

w. Water bath.

x. Thermo-stir hot plate.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

	y. Magnetic stirrer bar (Teflon). z. Thermometer, Celsius, 0-150° C range. aa. Knife, for cutting frozen tissue. bb. Sartorius electronic balance, model 3716, or equivalent. cc. Cahn electrobalance, model G-2, or equivalent. dd. Centrifuge, international size 2: model K, or equivalent.
2. Instrumentation	Gas liquid chromatograph: Hewlett Packard 5880, or equivalent, equipped with electron capture detector. Column 6 ft × 4 mm id packed with 3% OV-17 on Gas Chromosorb Q 100/120 mesh. Septum: high-temperature type (HT-13), Applied Science Laboratories, Inc., or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. N-hexane, glass distilled, Burdick and Jackson, or equivalent.

b. Toluene, glass distilled, Burdick and Jackson, or equivalent.

c. Ethyl acetate, glass distilled, Burdick and Jackson, or equivalent.

d. Acetone, glass distilled, Burdick and Jackson, or equivalent.

e. Potassium hydroxide, pellets, 85%, reagent, J. T. Baker, or equivalent.

f. Sodium hydroxide, pellets, reagent grade.

g. Sodium tetraborate decahydrate, reagent grade.

h. Boric acid, reagent grade.

i. 4-Fluoro-3-nitrobenzotrifluoride, INC Life Sciences Group (K & K), Plainview, NY.

j. Thymol blue indicator; Matheson, Coleman, and Bell; or equivalent.

k. 0.2N Hydrochloric acid.

l. 4M Potassium hydroxide; dissolve 264 g of 85% KOH in distilled water, dilute to 1 L.

m. 1M Sodium hydroxide.

n. 0.1M Sodium hydroxide.

o. 0.1M Sodium tetraborate.

p. 0.2M Boric acid.

q. 0.2% 4-Fluoro-3-nitrobenzotrifluoride: Dilute 0.135 mL (200 mg) of 4-fluoro-3-nitrobenzotrifluoride to 100 mL with distilled acetone. Fresh solutions are prepared. Use pipet control device (Adams, or equivalent) to deliver reagent.

r. 0.1% Thymol blue: Dissolve 100 mg of thymol blue in 100 mL of 0.1M sodium hydroxide.

s. Toluene-ethyl acetate (85:15): Dilute 150 mL of ethyl acetate to 1 L with toluene.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Morantel tartrate; pyrantel tartrate; N-ethyl-1, 3-propanediamine dihydrochloride; N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine; N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine —Pfizer, Inc.

2. Preparation of Standards

a. N-Ethyl-1, 3-propanediamine dihydrochloride internal standard solutions.

i. Stock solution A: Dissolve 42.5 mg reference standard N-ethyl-1, 3-propanediamine dihydrochloride in distilled water to dilute to 100 mL.

ii. Working standard solution B: Dilute 1.0 mL stock solution A to 25.0 mL with distilled water (conc. 17.0 μg/mL; 0.097 μmoles/mL).

iii. Working standard solution C: Dilute 5.0 mL of working standard solution B to 50.0 mL with distilled water (conc. 1.79 μg/mL; 0.097 μmoles/mL).

b. Morantel tartrate solutions.

i. Stock solution A: Dissolve 105.07 mg reference standard morantel tartrate (equivalent to 62.5 mg morantel) in distilled water and dilute to 100.0 mL (conc. 625 μg/mL as morantel; 2.84 μmoles/mL).

ii. Working standard solution B: Dilute 4.0 mL of stock solution A to 50.0 mL with distilled water (conc. 50.0 μg/mL as morantel; 0.227 μmoles/mL).

iii. Working standard solutions C, D, and E: Dilute 1.0, 2.0, and 4.0 mL of working standard solution B to 50 mL with distilled water (conc. 1.0, 2.0, and 4.0 μg/mL as morantel; 0.0045, 0.0091, and 0.018 μmoles/mL).

c. Pyrantel tartrate solutions.

i. Stock solution A: Dissolve 107.96 mg reference standard pyrantel tartrate (equivalent to 62.5 mg pyrantel) in distilled water and dilute to 100.0 mL (conc. 625.0 μg/mL as pyrantel; 3.034 μmoles/mL).

ii. Working standard solution B: Dilute 4.0 mL of stock solution A to 50.0 mL with distilled water (conc. 50 μg/mL as pyrantel; 0.2427 μmoles/mL).

iii. Working standard solution C: Dilute 20 mL of stock solution A to 50.0 mL with distilled water (conc. 50 μg/mL as pyrantel; 0.1214 μmoles/mL).

iv. Working standard solution D: Dilute 5 mL of working standard solution B to 50 mL with distilled water (conc. 5.0 μg/mL as pyrantel; 0.0243 μmoles/mL).

v. Working standard solution E: Dilute 5 mL of working standard solution C to 50 mL with distilled water (conc. 2.5 μg/mL as pyrantel; 0.0121 μmoles/mL).

DETERMINATIVE METHOD

D. STANDARDS (Continued)

d. N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine.

i. Stock solution A: Dissolve 5.15 mg of reference standard N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine in 200 mL toluene (stable if protected from light).

ii. Working standard solution B: Dilute 10.0 mL of stock solution A to 100.0 mL with toluene (conc. 2.58 μg/mL).

e. N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine.

i. Stock solution A: Dissolve 5.00 mg of reference standard N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine in 200 mL toluene (stable if protected from light).

ii. Working standard solution B: Dilute 10.0 mL of stock solution A to 100.0 mL with toluene (conc. 2.50 μg/mL).

f. Gas-Liquid chromatography calibration standard solutions for morantel in liver and pyrantel in muscle. Dilute 6.0, 5.0, 4.0, 3.0, and 2.0 mL aliquots respectively of N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine working standard solution B with 2.0 mL of N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine working standard solution B and adjust each solution to 250.0 mL with toluene. Cover flasks with aluminum foil. The concentrations of these derivatives are:

Solution Number	*N-methyl-1, 3-propane diamine derivative†*		*N-ethyl-1, 3 propane diamine derivative‡*		*Molar ratio: Methyl/ Ethyl*	*Equivalent ppm morantel§*
	μg/mL	*μmoles/mL*	*μg/mL*	*μmoles/mL*		
1	0.0600	1.29×10^{-4}	0.206	4.29×10^{-5}	3.0	1.2
2	0.0500	1.07×10^{-4}	"	"	2.5	1.0
3	0.0400	8.58×10^{-5}	"	"	2.0	0.8
4	0.0300	6.43×10^{-5}	"	"	1.5	0.6
5	0.0200	4.29×10^{-5}	"	"	1.0	0.4

† = N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine.

‡ = N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine.

§ = When the internal standard is added to tissue at a level equivalent to 0.4 ppm morantel.

g. Gas-liquid chromatography calibration standard solutions for pyrantel in liver. Dilute 4.0, 3.0, 2.0, and 1.0 mL aliquots respectively of N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine stock solution A with 2.0 mL of N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine stock solution A and adjust each solution to 250.0 mL with toluene. Cover flasks with aluminum foil. The concentrations of these derivatives are:

DETERMINATIVE METHOD

D. STANDARDS (Continued)

Solution Number	N-methyl-1, 3-propane diamine derivative† µg/mL	N-methyl-1, 3-propane diamine derivative† µmoles/mL	N-ethyl-1, 3 propane diamine derivative‡ µg/mL	N-ethyl-1, 3 propane diamine derivative‡ µmoles/mL	Molar ratio: Methyl/ Ethyl	Equivalent ppm pyrantel§
1	0.0600	1.29×10^{-3}	0.206	4.29×10^{-4}	3.0	12.0
2	0.0500	1.07×10^{-3}	"	"	2.5	10.0
3	0.0400	8.58×10^{-4}	"	"	2.0	8.0
4	0.0300	6.43×10^{-4}	"	"	1.5	6.0

† = N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-methyl-1, 3-propanediamine.

‡ = N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine.

§ = When the internal standard is added to tissue at a level equivalent to 0.4 ppm pyrantel.

g. Thin-layer chromatography marker solution: Dissolve 2.00 mg each of the bis-(2-nitro-4-trifluoromethylphenyl) derivatives of N-methyl- and N-ethyl-1, 3 propanediamine in 5.0 mL ethyl acetate.

3. Storage Conditions

Stock and working solutions of morantel tartrate, pyrantel tartrate, and N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine should be stored in the refrigerator.

4. Shelf Life Stability

Stock and working solutions of morantel tartrate, pyrantel tartrate, and N, N′-bis-(2-nitro-4-trifluoromethylphenyl)-N-ethyl-1, 3-propanediamine are stable for at least one month when properly stored.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Slice frozen tissue into small pieces, keeping it frozen during weighing.

2. Sample Extraction

a. Transfer 5.0 g of sliced tissue to a screw-cap test tube. Add 1.0 mL N-ethyl-1, 3-propanediamine dihydrochloride working internal standard solution B and 10 mL 4M potassium hydroxide solution. Cap the tube.

NOTE: To estimate morantel recoveries at the 0.2, 0.4, and 0.8 ppm levels, 5.0 g control sliced tissue is fortified with 1.0 mL morantel tartrate working standard solutions C, D, and E (1.0, 2.0, and 4.0 μg/mL as morantel base). To estimate pyrantel recoveries at the 1.88, 3.74, and 7.5 ppm levels, 5.0 g control sliced tissue is fortified with 1.0 mL pyrantel tartrate working standard solutions C, D, and E.

b. Immerse the tube in an oil bath at 110° C so that the contents of the tube are just below the oil level. Digest the samples for 16-18 hours (overnight). Cool the hydrolysates in an ice bath.

c. Pour the hydrolysate into a 50 mL centrifuge tube and add 6-7 g of potassium hydroxide pellets. Stopper, cool the sample, and dissolve the pellets by mixing on a test tube mixer. Return the tube to the ice bath.

d. Wash the test tube used for hydrolysis with 20 mL toluene and transfer the solvent to the centrifuge tube containing the hydrolysate. Stopper and shake gently for about 15 sec. Centrifuge the mixture at 2000-2500 rpm for 5 min to clarify the phases. Recover the toluene layer using a blow-out pipet equipped with a propipet bulb and transfer this extract to a 50 mL centrifuge tube.

e. Re-extract hydrolysate with an additional 20 mL toluene, centrifuge to clarify the extract, and combine the extracts. Discard the aqueous layer.

f. Add 5.0 mL of 0.2N HCl to the combined toluene extracts, shake for 10-15 sec, and centrifuge at 2000 rpm.

g. Aspirate the toluene layer and discard. Pipet 40 mL of the aqueous layer into a 50 mL centrifuge tube and add 1 drop of the thymol blue indicator. (Solution should be pink.) Add 0.5 mL of 0.2M boric acid and 1N sodium hydroxide until the color of the solution changes from pink to blue. Add 1 mL of 0.1M sodium tetraborate solution.

h. Add 5.0 mL of 0.2% 4-fluoro-3-nitro-benzotrifluoride solution, stopper, mix by gentle hand shaking, and place in a water bath set at 50-55° C for 16 hours (overnight).

i. Cool solution to room temperature. Add 5.0 mL hexane and 25 mL water. Mix on test tube mixer and centrifuge at 2000 rpm.

3. Thin-Layer Chromatography

a. Apply 50 μL of each of the hexane extracts to individual channels. Also prepare control channels by applying 50 μL of one of the sample hexane extracts. Let dry and overlay with 50 μL of chromatography marker solution. (Avoid developing plates in areas subject to drafts.)

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Prior to chromatographic development, place an edge (ca 5 mm deep) of the thin-layer plate into a beaker of ethyl acetate so that the solvent will rise through the applied sample zones to form them into narrow bands 1 cm above their origins. Air-dry the plate in a hood before chromatographic development.

c. Place the prepared plate in a chromatographic chamber lined with blotting paper and saturated with toluene-ethyl acetate (85:15). Develop until the solvent front reaches the top of the plate. Air-dry the developed plate in a fume hood to remove toluene.

d. Examine the plate under short wavelength ultraviolet light (254 nm) and locate on the control channel the zone containing the N, N′-bis-(2-nitro-4-trifluoromethylphenyl) derivatives of N-methyl-1, 3-propanediamine and N-ethyl-1, 3-propanediamine (R_f's ca 0.4 and 0.5 respectively).

e. Line up the tissue-related bands on the sample and control channels. Mark out a zone of silica gel on the sample channel encompassing an area containing each derivative.

NOTE: Allow for irregularities in the chromatographic migration of sample bands in zones to be removed by scraping along their periphery. Extracts of the derivative may be stored at room temperature for replication of the TLC step.

f. Using a 5 3/4″ Pasteur pipet with a glass wool plug, connect the narrow end to a vacuum supply and, with low vacuum, scrape and aspirate the selected zone up to the plug. Pour the powder into a 15 mL centrifuge tube and add 1.0 mL toluene. Drain the solvent into a centrifuge tube. Mix thoroughly and centrifuge. Examine the supernatant by gas-liquid chromatography.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions	a. Temperature. i. Column—260° C. ii. Injection port—280° C. iii. Detector—300° C. b. Carrier gas: argon/methane at 60 mL/min.
2. General Operation	Inject into the gas-liquid chromatograph equal μL aliquots of samples and GLC calibration solutions. The peak height of the ''EAPA'' component of these standards should approximate 30-40% of full-scale deflection. The retention times of the ''MAPA'' and ''EAPA'' components, under the recommended GLC conditions, are 4.7 and 3.8 min respectively.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

a. Compute the peak height (mm) ratio of the N-methyl to N-ethyl derivatives in each GLC calibration solution. Fit the data to the best straight line. Calculate the slope and the intercept for the standard curve using the peak height ratio as the ''Y'' coordinate and the equivalent ppm morantel or pyrantel level of the standards as the ''X'' coordinate.

b. From the standard slope and intercept values and the observed peak height ratio of the bis-(2-nitro-4-trifluoromethylphenyl) derivatives of N-methyl-1, 3-propanediamine and N-ethyl-1, 3-propanediamine in the sample, determine the ppm of morantel in the tissue sample by the following equation:

$$\text{ppm morantel in tissue} = \frac{\text{Peak height ratio of MAPA to EAPA in sample-intercept (Std)}}{\text{Slope (Std)}}$$

Determine the ppm of pyrantel in the tissue sample by the following equation.

$$\text{ppm pyrantel in tissue} = \frac{\text{Peak height ratio of MAPA to EAPA in sample-intercept (Std)}}{\text{Slope (Std)}}$$

2. References

a. Journal AOAC, Vol. 65, No. 2, 1982.

b. NADA 92-444, 93-903, and 43-290, Pfizer.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*
Morantel	0.2-0.8 (muscle) 0.4-1.2 (liver)	80-110	≤ 10
Pyrantel	0.4-1.2 (muscle) 6-12 (liver)	80-110	≤ 10

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Weight of sample	5.0 g ± 0.01 g
b. Weight of KOH	6.0-7.0 g
c. Temperature of water bath	50-55° C
d. Final volume of toluene	1.0 mL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—5 levels, 3 replicates each (each compound).

(a) Morantel: 0.4, 0.6, 0.8, 1.0, and 1.2 ppm.

(b) Pyrantel: 4.0, 6.0, 8.0, 10.0, and 12.0 ppm.

ii. Phase II: Fortified samples—5 levels, 3 replicates each (each compound) over a minimum of 3 days (30 total samples).

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples from FSIS Western Laboratory (samples submitted by supervisor if only one laboratory is performing this test).

(b) Report analytical findings to Chemistry Division.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst, or 20% of official samples analyzed (whichever is smaller).

ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All % recoveries.

(d) Running average, standard deviation, and CV for recoveries.

(e) Appropriate CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices.

i. Morantel—Bovine liver and muscle.

ii. Pyrantel—Swine liver and muscle.

b. Sample receipt size, minimum: Varied; enough to otain matrix for all required screening, quantitation, and confirmation tests, and reserve sample.

c. Condition upon receipt: Frozen.

d. Sample storage:

i. Time: Not yet determined.

ii. Condition: Frozen.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sensitivity	a. Lowest detectable level (LDL):
	b. Lowest reliable quantitation (LRQ):
	c. Minimum proficiency level (MPL): 0.5 ppm.

TRACE METAL ANALYSIS

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 7
F. Analytical Quantitation 9
G. Calculations 10
H. Hazard Analysis 11
I. Worksheet 12
J. Quality Assurance Plan 15

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

This method is appropriate for the analysis of copper (Cu), lead (Pb), zinc (Zn), cadmium (Cd), iron (Fe), nickel (Ni), cobalt (Co), and manganese (Mn) in animal tissues. These metals are obtained from the tissue by ashing at an elevated temperature in a suitable furnace. The sample matrix is first completely ashed in the furnace and then a second heat treatment is used to remove all remaining amounts of organic residue. The ash residue is dissolved in hydrochloric acid prior to analysis by atomic absorption (AA) or inductively coupled plasma (ICP) optical emission spectrometry.

2. Applicability

This method is suitable for the analysis of trace metals in the liver, kidney, or muscle tissue of all animal species.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Forced-air drying oven capable of maintaining a temperature of 95° C ± 5° C.

b. 50 mL ashing dishes, Vycor brand or equivalent (inner surfaces should not be etched).

c. Muffle furnace capable of maintaining a temperature of 550° C ± 10° C.

d. Hot plate capable of maintaining a surface temperature of 120° C ± 10° C.

e. Polyethylene or polypropylene centrifuge tubes, 15 mL.

f. 100 mL or 250 mL polyethylene bottles suitable for storing standard solutions.

g. 1000, 2000 mL volumetric flasks.

h. Repipet dispenser, 5 and 10 mL (Lab Industries #3005A, #3010A).

i. Teflon or polypropylene stirring rods.

2. Instrumentation

a. Atomic Absorption (AA) Spectrometry

Atomic absorption spectrometer equipped with background correction capability, appropriate multielement or single element hollow cathode lamp and an acetylene/air burner assembly. Perkin-Elmer model 603 or equivalent.

b. Inductively Coupled Plasma (ICP) Spectrometry

Inductively coupled plasma spectrometer—Perkin-Elmer model 5800 or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Nitric acid (HNO_3)—reagent grade, J. T. Baker, and redistilled, G. Frederick Smith Chemicals, or equivalent.

b. Hydrochloric acid (HCl)—reagent grade, J. T. Baker or equivalent.

c. Hydrochloric acid (1.00N aq. HCl). Dilute 83.0 mL of conc. HCl to 1000 mL with distilled deionized water, using a volumetric flask.

d. Magnesium nitrate hexahydrate solution (6.67% w/v, Solution 1): Dissolve 66.7 g $Mg(NO_3)2•6H_2O$ in 1000 mL of distilled deionized water.

e. Magnesium nitrate hexahydrate solution (3.33% w/v, Solution 2): Dissolve 33.3 g $Mg(NO_3)2•6H_2O$ in 1000 mL of 1N HCl.

f. 50% nitric acid (HNO_3). Dilute 500 mL redistilled nitric acid to 1000 mL with distilled deionized water.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

All standard solutions are made from commercially available reference standard solutions. Use Fisher Certified Atomic Absorption Standards (or equivalent) for all metal standards used in this procedure.

2. Preparation of Standards

a. Stock standards—Concentration of commercial standard solutions is typically 1000 μg/mL (i.e., 1000 ppm).

b. Calibration standards—Make dilutions as appropriate, using the 3.33% w/v aqueous magnesium nitrate solution to reach the following working concentrations. Check new standards versus old standards after proper calibration of the instrument. The new standards should assay to within 10% of the appropriate concentrations.

i. ICP calibration standards

Element	*Concentration (μg/mL)*
Cu	10.0
Pb	1.0
Zn	30.0
Cd	1.0
Fe	60.0
Ni	1.0
Co	1.0
Mn	1.0

ii. Atomic Absorption calibration standards

Standard #1

Element	*mL Stock Solution used*	*Final volume (mL)*	*Concentration (μg/mL)*
Zn	1	1000	1
Mn	1	1000	1
Fe	5	1000	5
Cu	5	1000	5
Cd*	1	1000	1

*NOTE: Separate standards for Pb and Cd are prepared because of frequent trace contamination from other metal standards. Make appropriate dilutions of the commercial standards using the 3.33% w/v aqueous magnesium nitrate solution.

Standard #2

Element	*mL Stock Solution used*	*Final volume (mL)*	*Concentration (μg/mL)*
Zn	3	1000	3
Mn	3	1000	3
Fe	15	1000	15
Cu	15	1000	15
Cd*	3	1000	3

*NOTE: Separate standards for Pb and Cd are prepared because of frequent trace contamination from other metal standards. Make appropriate dilutions of the commercial standards using the 3.33% w/v aqueous magnesium nitrate solution.

DETERMINATIVE METHOD

D. STANDARDS (continued)

Standard #3

Element	*mL of Stock Solution used*	*Final volume (mL)*	*Concentration (μg/mL)*
Zn	6	1000	6
Mn	6	1000	6
Fe	30	1000	30
Cu	30	1000	30
Ni	1	1000	1
Co	1	1000	1
Pb*	2	1000	2
Cd*	9	1000	9

*NOTE: Separate standards for Pb and Cd are prepared on account of frequent trace contamination from other metal standards. Make appropriate dilutions of the commercial standards using the 3.33% w/v aqueous magnesium nitrate solution.

c. Sample fortification solutions—Sample fortification solutions for ICP and AA are prepared from commercially available 1000 μg/mL certified standards. Add 1.0 mL of redistilled nitric acid per liter of solution.

i. Fortification solution #1. (Use for both AA and ICP.)

Element	*mL of Stock Solution used*	*Final volume (mL)*	*Concentration (μg/mL)*
Co	2	200	10
Ni	2	200	10
Mn	4	200	20
Cu	4	200	20

Use 1.0 mL of the above standards to fortify 15 g of the sample to yield the following concentrations:

Element	*Total μg*	*ppm*
Co	10	0.67
Ni	10	0.67
Mn	20	1.33
Cu	20	1.33

DETERMINATIVE METHOD

D. STANDARDS (Continued)

ii. Fortification solution #2. (Use for both AA and ICP.)

Element	*mL of Stock Solution used*	*Final volume (mL)*	*Concentration (μg/mL)*
Zn	60	250	240
Fe	60	250	240

Use 2.0 mL of the above standards to fortify 15 g of the sample to yield the following concentrations:

Element	*Total μg*	*Total ppm*
Zn	480	32
Fe	480	32

iii. Fortification solution #3. (Use for both AA and ICP.)

Element	*mL of Stock Solution used*	*Final volume (mL)*	*Concentration (μg/mL)*
Pb	10	200	50
Cd	10	200	50

Use 1.0 mL of the above solution to fortify 15 g of the sample to yield the following concentrations:

Element	*Total μg*	*Total ppm*
Pb	50	3.33
Cd	50	3.33

3. Storage Conditions

All standards should be stored at room temperature in clean polyethylene or polypropylene bottles with tightly secured caps.

4. Shelf LIfe Stability

Stock and calibration standards are stable for 2 years. All fortified standards should be prepared before use.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Muscle: Trim off as much fat as possible. Pass rapidly 3 times through a food chopper with plate openings no greater than 1/8". Mix thoroughly after each chopping.

b. Liver: Trim off as much connective tissue as possible. Place tissue into a blending jar and blend until the tissue is homogenized. Do not blend continuously for periods exceeding 1 minute. Excessive blending may overheat the liver tissue.

2. Ashing Procedure

a. Weigh 15.0 ± 0.1 g of sample tissue into a 50 mL Vycor crucible. Add 7.5 mL of 6.67% w/v magnesium nitrate solution and mix thoroughly.

b. Place the crucible in the drying oven, which should be set at 90°-95° C, until the sample is dry (approximately 6 hr). The sample must be dried thoroughly.

c. Place the sample in a cool (< 80° C) muffle furnace. Raise the temperature slowly (2°-4° C/min) to 350° C. Maintain at this temperature until the smoking ceases. Slowly raise the temperature to 500°-550° C. The sample must not be heated rapidly enough that it ignites. Hold at 500°-550° C for 16 hours (or overnight). Remove the sample from the oven and allow to cool to room temperature.

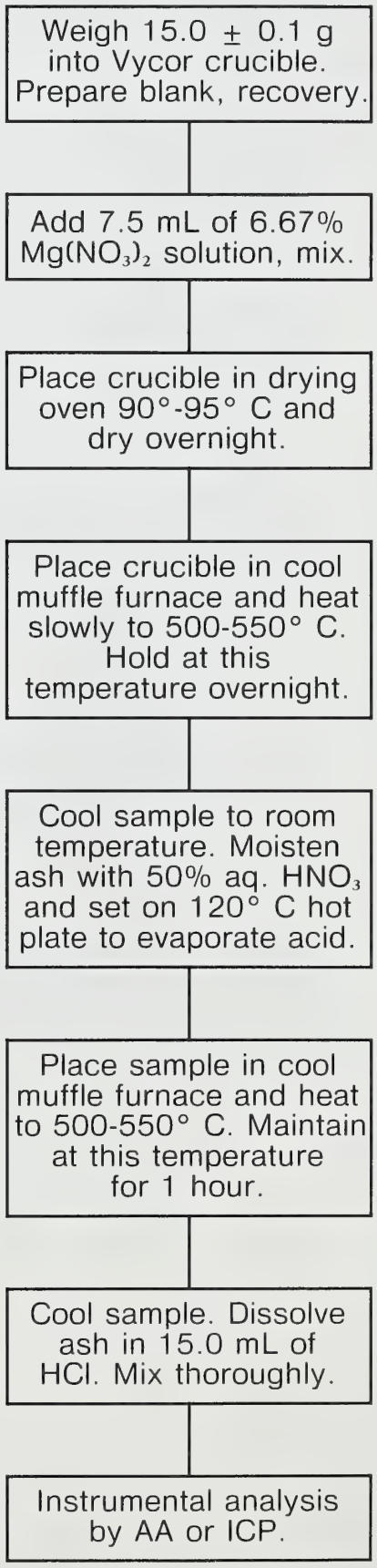
d. A second ashing step may be required to remove any remaining carbon residue. Add 2 mL 50% aq. HNO_3 while washing down the sides of the crucible. Place the sample on a hot plate set at 120° C to remove excess acid. Replace sample in the cool muffle furnace and raise to 500°-550° C. Maintain the sample at this temperature for 1 hr. Repeat this procedure until a carbon-free ash remains.

e. Completely dissolve the ash in 5.0 mL of 1.0N HCl. Transfer the dissolved sample to a polypropylene tube. Rinse the crucible with two additional 5.0 mL aliquots of 1.0N HCl and transfer to the same polypropylene tube to make a total volume of 15 mL. Cap tube and mix well.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Flow Chart Summary



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Instrumental Settings and Conditions

a. Atomic Absorption (AA) Spectrophotometry: Set up the AA spectrophotometer according to the manufacturer's instructions. Insert a copper hollow cathode tube lamp and maximize the signal intensity by adjusting the position of the lamp. Make a fine tuning of the wavelength by maximizing the signal. Adjust the height of the burner. Ignite the flame and aspirate a copper standard (e.g., 5 μg/mL) into the flame. Adjust the aspiration rate and position of the burner head for maximum signal.

b. Inductively Coupled Plasma (ICP) Spectrometry: Set up the ICP according to manufacturer's instructions. Adjust torch position for the maximum response of manganese. Perform a background equivalence check (BEC) and a coefficient of variation (CV) test for the manganese calibration standard (i.e., 1.0 μg/mL Mn). The BEC should be < 0.05 and the CV $\leq 1.0\%$ for ten replicates (or within manufacturer's specifications).

The following table provides an example of the operating conditions (using the Perkin-Elmer model 5800 ICP) for wavelength, forward power level, nebulizer flow rate, and viewing height above the load coil per element. Torch flow is 15 L/min argon and the auxiliary flow is adjusted to 1.0 L/min. A cross-flow nebulizer is used with a pump rate (sample uptake) of 1 mL/min aqueous solution and a nebulizer flow of 1.0 L/min argon.

Element	*Wavelength (nm)*	*Power level (watts)*	*Nebulizer flow (L/min)*	*Viewing height (nm)*
Cu	327.4	1240	1.07	15
Pb	220.3	1600	0.83	11
Zn	213.9	1360	0.86	12
Cd	214.4	1500	0.70	11
Fe	233.3	1300	1.07	14
Ni	231.6	1500	0.87	12
Co	230.7	1600	0.76	10
Mn	257.6	1300	0.96	16

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Element concentrations are calculated directly by the AA or ICP spectrometers. With other types or models of instrumentation, it may be necessary to construct a standard curve for each metal.

Optional calculation using the standard curve constructed manually:

$$\text{ppm (analyzed)}: \frac{\mu\text{g (from curve)} \times \text{mL (final volume)}}{\text{g (sample weight)}}$$

$$\text{ppm (actual)}: \frac{\text{ppm analyzed}}{\text{running 10 avg. recoveries}}$$

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination of Trace Metals in Animal Tissue by Atomic Absorption or Inductively Coupled Plasma Optical Emission Spectrometry

2. Required Protective Equipment

Safety glasses, plastic gloves, laboratory jacket or coat, heat-resistant gloves, and crucible tongs.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
B. Equipment		
Muffle furnace	Hot surfaces.	Use crucible tongs and heat-resistant gloves to insert or remove crucibles from hot furnace.
Acetylene	Explosive if mixed with air. Explosive if line pressure flow regulator to instrument exceeds 15 psi.	Check gas flow and system before flame ignition. Check tank pressure. Do not let it drop below 80 psi to avoid acetone entering gas line.
C. Reagents		
Nitric acid Hydrochloric acid	Corrosive. Contact with fumes or liquid can result in burns and severe skin, eye, and respiratory irritation.	All steps using acids should be performed in a fume hood while wearing plastic gloves.

4. Disposal Procedure

Sample solution, dilute standards, reagents.	Same as in procedure steps.	All solutions can be flushed down the drain with lots of water.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Metal Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

TRACE METAL ANALYSIS
BY ATOMIC ABSORPTION/ICP SPECTROPHOTOMETRY

ANALYST _____

DATE STARTED _____

DATE COMPLETED _____

		YES	NO
Section E.2.c.	500-550° C.	☐	☐
Section E.2.e.	15.0 mL	☐	☐

Sample ID	Tissue Code	Sample Wt.	Cu 403	Pb 404	Zn 405	Cd 406	Fe 411	Ni 412	Co 413	Mn 414				
CONTROL														
FORTIFIED														
% RECOVERY														
RUN 10 AVG REC														

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Element	*Analytical Range (μg/mL)*		*Acceptable Recovery (%)*	*Repeatability %CV*
	AA	ICP	AA and ICP	
Cu	0-30	0-10	70-110	≤ 15
Pb	0-2.0	0-3.0	70-110	≤ 15
Zn	0-6.0	0-30	70-110	≤ 15
Cd	0-9.0	0-1.0	70-110	≤ 15
Fe	0-30	0-60	70-110	≤ 15
Ni	0-1.0	0-1.0	70-110	≤ 15
Co	0-1.0	0-1.0	70-110	≤ 15
Mn	0-6.0	0-1.0	70-110	≤ 15

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Weight of sample	15.0 ± 0.1 g
b. Muffle furnace—final temperature	500°-550° C
c. Final volume	15.0 ± 0.5 mL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Triplicate standard curves for AA and ICP on 3 consecutive days.

ii. Phase II: Fortified samples—Triplicate analyses on fortified samples at 0, ½, 1, and 2 times the concentration of interest on 3 different days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to Section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Initially a minimum of 1 check sample per set, reduced to once per week per analyst. This sample is an internal check sample. It is a pooled sample analyzed at least 10 times to obtain a running average.

ii. Records are maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on differences between replicates.

(c) All percent recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver, kidney, muscle.

b. Sample receipt size: Approximately 50 g.

c. Condition upon receipt: Not spoiled or rancid.

d. Sample storage:

i. Time: 6 months

ii. Condition: Frozen ($< -10°$ C)

6. Sample Set

Each sample set must include a reagent blank, a blank tissue, a blank tissue fortified at the concentration level of interest, and up to 20 samples.

NOTE: Reagent blank is required to help determine if there is any trace of contamination from glassware or reagent used.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

7. Sensitivity

a. Lowest detectable level (LDL): The levels listed below are suggested by the manufacturer, not determined experimentally.

Element	*LDL (AA) (μg/mL)*	*LDL (ICP) (μg/mL)*
Cu	0.001	0.003
Pb	0.01	0.05
Zn	0.001	0.002
Cd	0.0005	0.004
Fe	0.003	0.003
Ni	0.004	0.01
Co	0.006	0.006
Mn	0.001	0.001

b. Lowest reliable quantitation (LRQ):

Element	*LRQ (AA) (μg/mL)*	*LRQ (ICP) (ppm)*
Cu	0.09	0.002
Pb	0.5	0.050
Zn	0.018	0.006
Cd	0.025	0.002
Fe	0.12	0.140
Ni	0.15	0.014
Co	0.15	0.012
Mn	0.055	0.002

c. Minimum proficiency level (MPL): Not determined.

NARASIN IN CATTLE AND CHICKEN TISSUES

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 10
G. [Reserved]
H. [Reserved]
I. [Reserved]
J. [Reserved]

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Narasin is extracted from samples with methanol in the case of lean, liver, or kidney and redistilled hexane in the case of skin or fat (skin not applicable to cattle). Tissue extracts are processed by carbon tetrachloride extraction in the case of lean, liver, or kidney and acetonitrile in the case of skin or fat. Subsequently all samples are purified by silica gel column chromatography and alumina column chromatography. Aliquots of the semipurified extracts in methanol are subjected to thin-layer chromatography on silica gel plates, and the antibiotic is detected on the TLC plates by bioautographic techniques using *Bacillus subtilis* as the assay organism. Narasin levels are estimated by comparing zones of inhibition from experimental samples to those from standard recoveries. Samples are authenticated by Polaroid photography.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

a. Tissue blender: Hamilton Beach Model 266, or equivalent, equipped with blender heads to fit a one-pint Ball Mason jar.

b. Centrifuge: International Centrifuge Model V, size 2, or equivalent.

c. Rotary vacuum evaporator: Rinco evaporator, or equivalent.

d. Thin-layer chromatography spreading board, applicator, and developing chambers: Brinkmann Instruments, Inc., Westbury, NY, or equivalents.

e. Photographic equipment: Polaroid Model MP3, or equivalent, and Type 55 Polaroid film.

f. Incubator maintained at 37° C.

g. Colorimeter: Bausch and Lomb Spectronic-20, or equivalent.

h. Water bath.

i. Grumbacher atomizer No. 836 sprayer, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane, A.R., redistilled.

b. Methanol A.R.

c. Chloroform A.R.

d. Benzene A.R.

e. Carbon tetrachloride A.R.

f. Ethylene glycol monomethyl ether A.R.

g. Acetonitrile.

h. Chloroform A.R., containing 5% by volume of methanol A.R.

i. Chloroform A.R., containing 1% by volume of methanol A.R.

j. Silica gel 0.2 to 0.5 mm for column chromatography: Brinkmann Instruments, Inc.

k. Alumina, Alcoa F-20, for column chromatography.

l. Glass wool.

m. Sodium sulfate, anyhdrous, A.R.

n. Agar medium No. 23: Dissolve 0.69 g $K_2HPO_4 \bullet 3H_2O$, 0.45 g KH_2PO_4, 2.5 g yeast extract (Difco), 10 g glucose (cerelose), and 6.0 g agar-agar (Colab Ionagar No. 2S) in enough deionized water to give 1 L total volume. Autoclave the solution for 15-20 min at 121° C.

o. Thin-layer chromatography plates: Prepare a slurry of 32 g silica gel G in 74 mL of deionized water and apply to standard 20 × 20 cm glass plates at a thickness of 250 microns. Air-dry the plates for 10 to 15 min. Activate the plates in a drying oven by heating at 100° C for 1 hr. Following activation, allow the plates to cool slowly to room temperature.

p. Alternatively, commercial silica gel thin-layer chromatographic plates may be used, such as Quanta Q47G (Quantum Industries) or Analtech made with Woelm silica gel (Analtech, Inc.).

q. *Bacillus subtilis* stock suspension.

   i. Wash the growth of *Bacillus subtilis*, American Type Culture Collection No. 6633, from one penassay seed agar slant (culture medium No. 1, from Grove and Randall, Assay Methods of Antibiotics) with 3 to 5 mL sterile deionized water onto the surface of a Roux bottle containing 300 mL of penassay seed agar with 0.33% manganous sulfate added.

   ii. Incubate the culture for 1 week at 37° C.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

iii. Following incubation, wash the growth from the agar surface of the Roux bottle with approximately 50 mL of sterile deionized water.

iv. Transfer the wash to a sterile 250 mL centrifuge bottle and hold the organisms at 65° C in a water bath for 30 minutes.

v. Centrifuge the suspension and discard the supernatant liquid.

vi. Repeat the resuspension and discard the supernatant liquid.

vii. Repeat the resuspension and washing of the organisms three times.

viii. After the final wash, heat-shock the cells again by immersion in the 65° C water bath for 30 min and suspend them in 30 mL sterile deionized water.

NOTE: This stock suspension is stored at 4°-5° C for use.

r. *Bacillus subtilis* inoculum: Prepare the inoculum fresh daily by diluting the stock suspension with sterile deionized water to obtain a 20% light transmittance at 530 nm using a Spectronic-20 colorimeter.

s. A 2 mg/mL solution of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride).

t. Sodium bicarbonate A.R.

u. Deacidified methanol A.R.: Add 1.0 g sodium bicarbonate A.R. to 100 mL methanol A.R. and mix. Filter off the excess sodium bicarbonate through Whatman No. 2 filter paper.

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

Standard solutions.

a. Weigh a quantity of narasin standard to contain 10 mg of narasin activity.

b. Transfer the standard quantitatively to a 100 mL volumetric flask and dissolve it in deacidified methanol. Dilute to the mark with deacidified methanol and mix thoroughly. This standard contains 100 μg/mL of narasin activity.

c. Pipette 5 mL of 100 μg/mL standard into a 50 mL volumetric flask with a volumetric pipette. Dilute to the mark with deacidified methanol and mix thoroughly. This standard contains 10 μg/mL of narasin activity.

d. Pipette 5 mL of 10 μg/mL standard into a 50 mL volumetric flask with a volumetric pipette. Dilute to the mark with deacidified methanol and mix thoroughly. This standard contains 1.0 μg/mL of narasin activity.

2. Storage Conditions

Stock solutions should be stored tightly stoppered at 4° C.

3. Shelf Life Stability

Standard soluitions 2.b.-2.d are stable at least 5 days when stored under refrigeration as described in section 3.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Lean, Liver, and Kidney Tissue Extraction and Purification

a. Weigh 50 g ground or blended tissue sample into a one-pint extraction jar. Prepare negative control samples and standard recoveries for assay with each set of samples.

   i. Weigh appropriate control tissue into one-pint blender jars.

   ii. Add standard narasin (1 μg/mL in methanol) by pipette to give the desired concentration in the recovery samples.

   NOTE: One negative control (no narasin added), one recovery sample containing 5 ng/g wet tissue (5 ppb), one recovery sample containing 10 ng/g (10 ppb), and one recovery sample containing 20 ng/g (20 ppb) are processed with each set of the samples.

   Control and recovery samples are extracted and chromatographed exactly as described for the corresponding tissue.

b. Add 2 mL methanol per gram of tissue.

c. Blend sample until uniform (approximately 30 sec).

d. Transfer sample to 250 mL centrifuge bottle and centrifuge for 10 min at 2500 rpm.

e. Transfer the supernate (measure via a graduated cylinder) to a 250 mL separatory funnel.

f. Extract the supernate with three 25 mL potions of carbon tetrachloride. Combine the carbon tetrachloride fractions in a 125 mL evaporating flask and evaporate to dryness by rotary vacuum evaporation, using a water bath at 50°-60° C.

g. Prepare silica gel column for chromatography. Prepare a separate column for each sample.

   i. Place approximately 10 mL of chloroform into a 14 X 240 mm glass chromatographic column. Insert a glass wool pledget and tamp with a glass stirring rod to eliminate air bubbles.

   ii. Add 10 mL (measured with a 10 mL graduated cylinder) of 0.2 to 0.5 mm silica gel to the column and follow with 5 mL chloroform.

   iii. Stir the silica gel with a glass rod to eliminate air bubbles; then let the column stand until the silica gel settles.

   iv. Add approximately 5 mL (measured with a 5 mL graduated cylinder) of anhydrous sodium sulfate to the column, layering it carefully to avoid disturbance of the silica gel surface.

   v. Drain the chloroform to the top of the sodium sulfate.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

h. Dissolve the sample from step f in 20 mL of chloroform and charge the silica gel column with solution at a flow rate of 3-4 mL per min. Rinse the evaporating flask with additional 20 mL and 10 mL aliquots of chloroform and transfer the rinses to the column. Discard the chloroform effluent.

i. Develop the silica gel column with 200 mL of chloroform at a flow rate of approximately 4-6 mL/min. Discard the chloroform effluent.

j. Place an evaporating flask into position to receive the column effluent.

k. Elute the narasin from the column with 50 mL of chloroform:methanol (95:5) at a flow rate of 3-4 mL/min.

l. Evaporate the eluate to dryness by rotary vacuum evaporation using a water bath at 50°-60° C.

m. Prepare an alumina column for chromatography.

Prepare a separate column for each sample.

i. Place approximately 10 mL of chloroform into a 14 × 250 mm glass chromatographic column. Insert a glass wool pledget and tamp with a glass stirring rod to eliminate air bubbles.

ii. Add 5 mL of Alcoa F-20 chromatographic alumina (measured with a 5 mL graduated cylinder) and follow with 5 mL of chloroform. Be sure alumina has been washed from the sides of the column. Let the column stand until the alumina settles.

iii. Add approximately 2 mL of anhydrous sodium sulfate (measured with a 5 mL graduated cylinder) to the column, layering it carefully to avoid disturbance of the alumina surface.

iv. Drain the chloroform to the top of the sodium sulfate.

n. Place an evaporating flask into position to receive the column effluent.

o. Dissolve the sample from step l in 20 mL of chloroform:methanol (99:1) and charge the alumina column with this solution at a flow rate of 3-4 mL/min. Rinse the evaporating flask with additional 20 mL and 10 mL aliquots of chloroform:methanol (99:1) and transfer the rinses to the column. Collect the chloroform:methanol effluent.

p. Evaporate the eluate to dryness and transfer the sample to a 15 mL screw-cap sample bottle in 5 mL of redistilled hexane. Rinse the flask with an additional 5 mL of hexane and transfer the rinse to the sample bottle.

q. Evaporate the hexane under a mild stream of compressed air.

r. Dissolve the sample in methanol, using 0.02 mL methanol per 10 mL of supernate extracted.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Skin Tissue Extraction and Purification

a. Weigh a ground or blended 50 g tissue sample into a one-pint extraction jar.

b. Add 4 mL of redistilled hexane per gram of tissue and place on the steam bath until the tissue is dissolved or is dissolved as much as possible. Stir occasionally to facilitate dissolution. About 15 to 20 min will usually suffice.

c. Centrifuge the sample 10-15 min at 2500 rpm.

d. Transfer the supernate (measure via a graduated cylinder) to a 500 mL separatory funnel.

e. Extract the supernate with five 1 mL/g tissue portions of acetonitrile. Combine the acetonitrile fractions in a 500 mL evaporating flask and evaporate to dryness by rotary vacuum evaporator, using a water bath at 50°-60° C.

f. Continue as described in steps 1.g-1.q above.

g. Dissolve the sample in methanol, using 0.01 mL methanol per 10 mL of supernate.

3. Fat Tissue Extraction and Purification

a. Perform steps described in 2.a-2.c above.

b. Transfer the supernate to a 500 mL separatory funnel.

c. Perform steps described in steps 2.e and 1.g-1.q above.

d. Dissolve the sample in 0.004 mL methanol/g tissue.

4. Thin-Layer Chromatography

Chromatograph the extracts from 1, 2, and 3 above on thin-layer plates as follows:

a. Apply 20 μL of methanol extract of negative control sample, standard recovery samples, and experimental samples to silica gel thin-layer plate. A forced-air hair dryer may be used to hasten evaporation of solvent during sample application.

NOTE: Up to 9 samples (1 negative control, 3 recoveries, and 5 samples) may be assayed on one thin-layer plate.

b. Add negative control and recovery samples to each thin-layer plate in order to ensure adequate standardization of the procedure.

c. Label the TLC plate and draw a line in the silica gel with a pencil on a scribe 16 cm from the bottom of the plate to mark the solvent front. Center the applied samples 1.5 cm from the bottom of the plate.

d. Line a TLC developing chamber with a sheet of filter paper or white blotter paper to improve saturation of the atmosphere in the chamber.

e. Prepare the developing solvent by mixing 80 parts carbon tetrachloride with 10 parts benzene and 6 parts ethylene glycol monomethyl ether.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

f. Add the solvents to the developing chamber prior to placing the filter (or blotter) paper in the tank. Prepare the tank 1 to 2 hr prior to introduction of the thin-layer plates in order to ensure saturation of the paper and atmosphere.

g. Develop the thin-layer plate in the chamber, allowing the solvent front to move up to the line previously scored on the plate.

h. Remove the plate from the chamber and allow the plate to air-dry on the laboratory bench.

5. Preparation of Bioautographs

Bioautographs are prepared according to the method of Kline and Golab, *J. Chromatog.*, 18, 409 (1965).

a. Melt No. 23 agar in a steam bath.

b. Pour approximately 50 mL of melted agar into a 250 mL Erlenmeyer flask and fix the TLC plate by spraying the surface with the agar, using an artist's atomizer-type sprayer (Grumbacher atomizer No. 836) attached to the laboratory compressed air supply.

c. Place the TLC plate in a plexiglass holder (or other suitable container that will prevent contamination of the plate) and allow the plate to come to room temperature.

NOTE: This step is necessary to prevent too rapid solidification of the seeded agar overlay.

d. Cool 60 mL of melted No. 23 agar to 52° C in a 125 mL Erlenmeyer flask.

NOTE: Cool slowly to prevent solidification of the agar before it can be inoculated and poured.

e. Inoculate the agar with 0.2% of *B. subtilis* inoculum (refer to section C, Reagent and Solution List, item r). Mix quickly by swirling.

f. Pour this seeded agar over the surface of the TLC plate.

NOTE: Pouring of the seed agar must be done rapidly and carefully to ensure an even agar overlay before the agar solidifies. Also, the closer to using only 40 mL of the agar to pour the bioautograph, the greater the sensitivity.

g. Allow the plate to cool until the agar sets.

h. Cover the TLC plate holder to prevent contamination and to prevent evaporation of moisture from the plate.

i. Incubate the bioautograph overnight (16-18 hr) at 37° C.

j. Subsequent to incubation, spray the plate with a solution of INT (refer to section C, Reagent and Solution List, item s). Allow the color to develop for a period of 1-2 hr. An additional spraying applied after the first spraying has been absorbed into the agar is helpful in increasing the rate and degree of color contrast.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Estimation of Concentration

a. After incubation, locate the narasin zones on the bioautograph at the R_f of 0.3 to 0.4.

b. Estimate narasin concentration by visually comparing zone sizes from experimental samples to zone sizes from standard recovery samples.

   i. Negative samples are reported as no activity at a sensitivity of the lowest positive standard recovery.

   ii. Positive samples are reported as the concentration of the corresponding standard recovery level or as a range between two standard recoveries.

   NOTE: Narasin levels obtained in this manner are regarded as approximate rather than absolute values.

c. If permanent records of bioautographs are desired, label the plates for identification and photograph by either transmitted or reflected light.

   NOTE: Polaroid photography of bioautographs has the advantage of producing a print immediately so that a satisfactory print may be obtained before destruction of the bioautograph.

d. Test sensitivity when standard narasin is applied to the TLC plates in methanol solution is 30 to 50 ng.

e. Test sensitivity of standard recoveries processed by the extraction procedures described is approximately 5 ng narasin per gram of fresh tissue (5 ppb).

f. The assay must be regarded as semiquantitative and is more applicable to verifying absence of narasin than to exact measurement of narasin levels.

2. References

Ely LIlly and Co. Procedure 5801652 (1975).

Elanco, NADA 118-980.

Kline and Golab, *J. Chromatog.*, 409 (1965).

NOVOBIOCIN AND VIRGINIAMYCIN

Contents

A. Introduction 1
B. Equipment 3
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 7
F. Analytical Quantitation 11
G. Calculations 20
H. Hazard Analysis 21
I. Worksheet 22
J. Quality Assurance Plan 25

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structures

The antibiotic compounds novobiocin (NOV) and virginiamycin (VIR) are used in the treatment of meat-producing animals. This method was developed to provide chemical identification of the antibiotics. Additionally, the method provides quantitative supporting data for the official biological method.

Residues are extracted from tissue by blending with methanol. The homogenate is centrifuged to remove solid material and an aliquot of the organic extract is removed and filtered for HPLC analysis of novobiocin. A second aliquot is transferred to a clean tube and processed for analysis of virginiamycin by extraction into dichloromethane. Both compounds are determined in a single gradient elution HPLC analysis on an LC-18-DB reversed phase LC column.

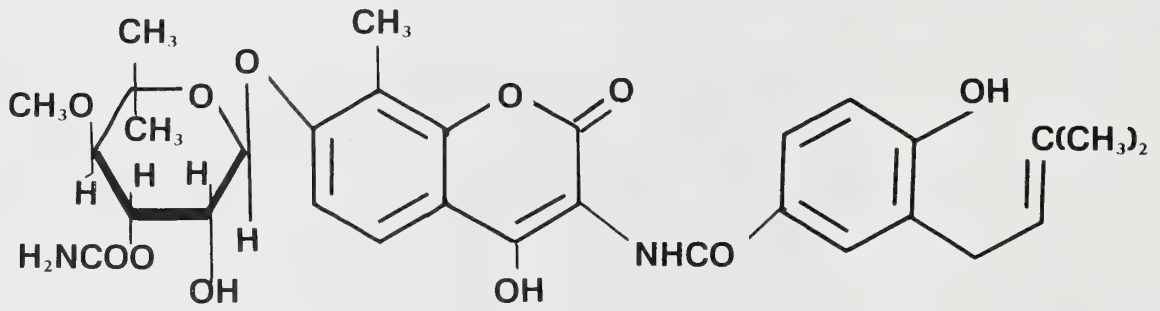
2. Applicability

This method is applicable to bovine, porcine, and avian species, and liver, muscle, or kidney tissue.

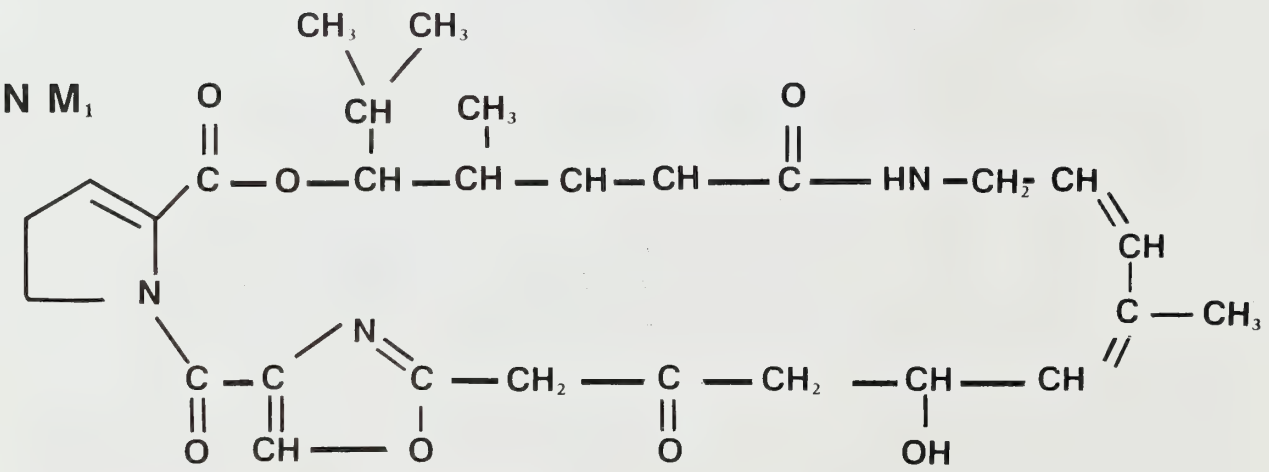
DETERMINATIVE METHOD

A. INTRODUCTION (Continued)

NOVOBIOCIN



VIRGINIAMYCIN M_1



DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Mechanical shaker: Eberbach reciprocating flat bed linear shaker.

b. N-Evap solvent removal system: Organomation Associates, Inc., Model 112, 24 place, or equivalent.

c. Centrifuge: Sorvall Model T600B, or equivalent.

d. Tissuemizer: Polytron Tissue Homogenizer, or equivalent.

e. 50 mL polypropylene centrifuge tubes: Falcon Blue Max, #2070, or equivalent.

f. Disposable syringe filters: Applied Science Series 6000 PTFE, 0.2 μm porosity, or equivalent.

2. Instrumentation

HPLC System: Hewlett Packard 1090 HPLC System equipped as follows:

i. Hewlett Packard Diode Array UV Detection System.

ii. Chem Station control hardware and software.

iii. Automatic injection system.

iv. Supelco LC-18 DB column, 15 cm × 4.6 mm i.d., 5 μm particle size (Supelco, Inc., catalog no. 5-8348888).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Methanol, Burdick and Jackson HPLC Grade, or equivalent.

b. Petroleum ether, Baker Resi-Analyzed Grade, or equivalent.

c. Methylene chloride (dichloromethane), Baker Resi-Analyzed Grade, or equivalent.

d. Acetonitrile, Burdick and Jackson HPLC Grade, or equivalent.

e. Phosphoric acid, Mallinckrodt Reagent Grade 85% (catalog no. 2761-1), or equivalent.

f. Ammonium phosphate, Mallinckrodt Reagent Grade (catalog no. 3476), or equivalent.

2. Solution List

a. 0.2M ammounium phosphate.

b. 0.01M phosphoric acid.

c. Acetonitrile:0.01M phosphoric acid—20:80.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Novobiocin: Sigma Chemical Catalog No. N-1628, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO.

b. Virginiamycin-M1: Sigma Chemical Cat. No. V-2753, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO.

2. Preparation of Standards

a. Stock standard solutions (50 μg/mL).

i. Novobiocin—Accurately weigh 5.0 $\pm$ 0.1 mg noviobiocin reference standard material into a clean 100 mL volumetric flask. Dissolve and dilute to volume with HPLC-grade methanol.

ii. Virginiamycin—Accurately weigh 5.0 $\pm$ 0.1 mg virginiamycin reference standard material into a clean 100 mL volumetric flask. Dissolve and dilute to volume with HPLC-grade methanol.

b. Fortification standards.

i. Novobiocin—Stock standard solution will be used.

ii. Virginiamycin—Pipet 20 mL virginiamycin stock standard solution into a clean 100 mL volumetric flask and dilute to volume with HPLC-grade methanol to produce fortification standard solution. Concentration is 10 μg/mL.

c. HPLC reference standards.

i. Novobiocin.

(a) Pipet 20 mL novobiocin stock standard solution into a clean 100 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 10 μg/mL.

(b) Pipet 1.66 mL novobiocin solution (a) above into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.166 μg/mL, equivalent to 0.5 ppm tissue concentration.

(c) Pipet 3.33 mL novobiocin solution (a) above into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.333 μg/mL, equivalent to 1.0 ppm tissue concentration.

(d) Pipet 6.66 mL novobiocin solution (a) above into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.666 μg/mL, equivalent to 2.0 ppm tissue concentration.

ii. Virginiamycin.

(a) Pipet 2 mL virginiamycin fortification standard solution into a clean 50 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 0.4 μg/mL, equivalent to 0.1 ppm tissue concentration.

DETERMINATIVE METHOD

D. STANDARDS (Continued)

	ii. Pipet 4 mL virginiamycin fortification standard solution into a clean 50 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 0.8 μg/mL, equivalent to 0.2 ppm tissue concentration. iii. Pipet 8 mL virginiamycin fortification standard solution into a clean 50 mL volumetric flask and dilute to volume with HPLC-grade methanol. Concentration is 1.6 μg/mL, equivalent to 0.4 ppm tissue concentration.
3. Storage Conditions	Stock solutions should be stored tightly stoppered at 4° C.
4. Shelf Life Stability	Stock solutions are stable up to 6 months if stored as described above.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Procedure

a. Weigh 5.0 ± 0.1 g homogenized tissue into a clean 50 mL polypropylene centrifuge tube.

NOTE: Recovery samples for novobiocin are fortified using the novobiocin stock standard solution as follows: 1.0 ppm tissue level, add 100 μL novobiocin stock solution.

Recovery samples for virginiamycin are fortified using the virginiamycin fortification standard solution as follows: 0.2 ppm tissue level, add 100 μL virginiamycin fortification solution.

b. Add 15 mL HPLC-grade methanol and blend using a Polytron Tissuemizer or similar blending device at medium speed for 1 minute.

c. Centrifuge samples for 5 minutes at 2000 rpm (1000 × G).

d. Remove 1 mL of the clear methanol layer and pass through a 0.2 μm syringe filter, collecting filtrate in an HPLC auto sampler vial. Cap sample and reserve for analysis of novobiocin by HPLC.

e. Remove 6 mL of the clear methanol layer and place in a clean 50 mL polypropylene centrifuge tube. Add 5 mL 0.2M $NH_4H_2PO_4$ and 5 mL petroleum ether. Cap samples and shake vigorously for 30 seconds using a flat-bed reciprocating linear shaker.

f. Centrifuge for 5 minutes at 2000 rpm (1000 × G), aspirate, and discard the petroleum ether layer.

g. Add 5 mL petroleum ether and 25 mL dichloromethane, cap, and shake vigorously for 2 minutes, using mechanical shaker.

h. Aspirate and discard the upper organic layer.

i. Add 0.6 mL distilled water and 0.6 mL acetonitrile. Reduce volume to approximately 0.3 mL, using an N-Evap solvent evaporation device with water bath set at 50° C and a gentle stream of dry nitrogen.

j. Add 12 mL HPLC-grade acetonitrile, carefully rinsing the sides of the tube. Evaporate to dryness using the N-Evap.

k. Redissolve residue in 500 μL of the HPLC mobile phase (20:80 acetonitrile:0.01M phosphoric acid) and transfer to an HPLC autosampler vial. Cap samples and reserve for analysis by HPLC.

2. Screening Test

Since it is anticipated that the vast majority (approaching 100%) of all samples analyzed by this method will be negative, it is efficient to screen all sample extracts prior to quantitative analysis. To perform the screening test, the following sequence should be followed:

a. Inject 25 μL of the novobiocin/virginiamycin mixed HPLC reference standard, followed by the novobiocin sample extracts and then the virginiamycin sample extracts.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

b. Evaluate the resulting chromatograms for tentative positive findings. A sample is identified as tentatively positive if both of the following conditions are met:

i. There is a peak within ± 0.1 min of the retention time of either the novobiocin or virginiamycin reference peak from the standard injection.

ii. The peak in question is ≥ 20% of the peak height of the corresponding reference standard.

3. Preparation of Standard Curve

If both of the conditions in E.2.b are met, the sample is identified as tentatively positive. If such a case exists, the following quantitative determination should be performed in order to produce a standard curve:

a. Inject 25 μL of the appropriate low level HPLC standard (0.166 μg/mL for novobiocin and 0.4 μg/mL for virginiamycin).

b. Inject 25 μL of the appropriate middle level HPLC standard (0.333 μg/mL for novobiocin and 0.8 μg/mL for virginiamycin).

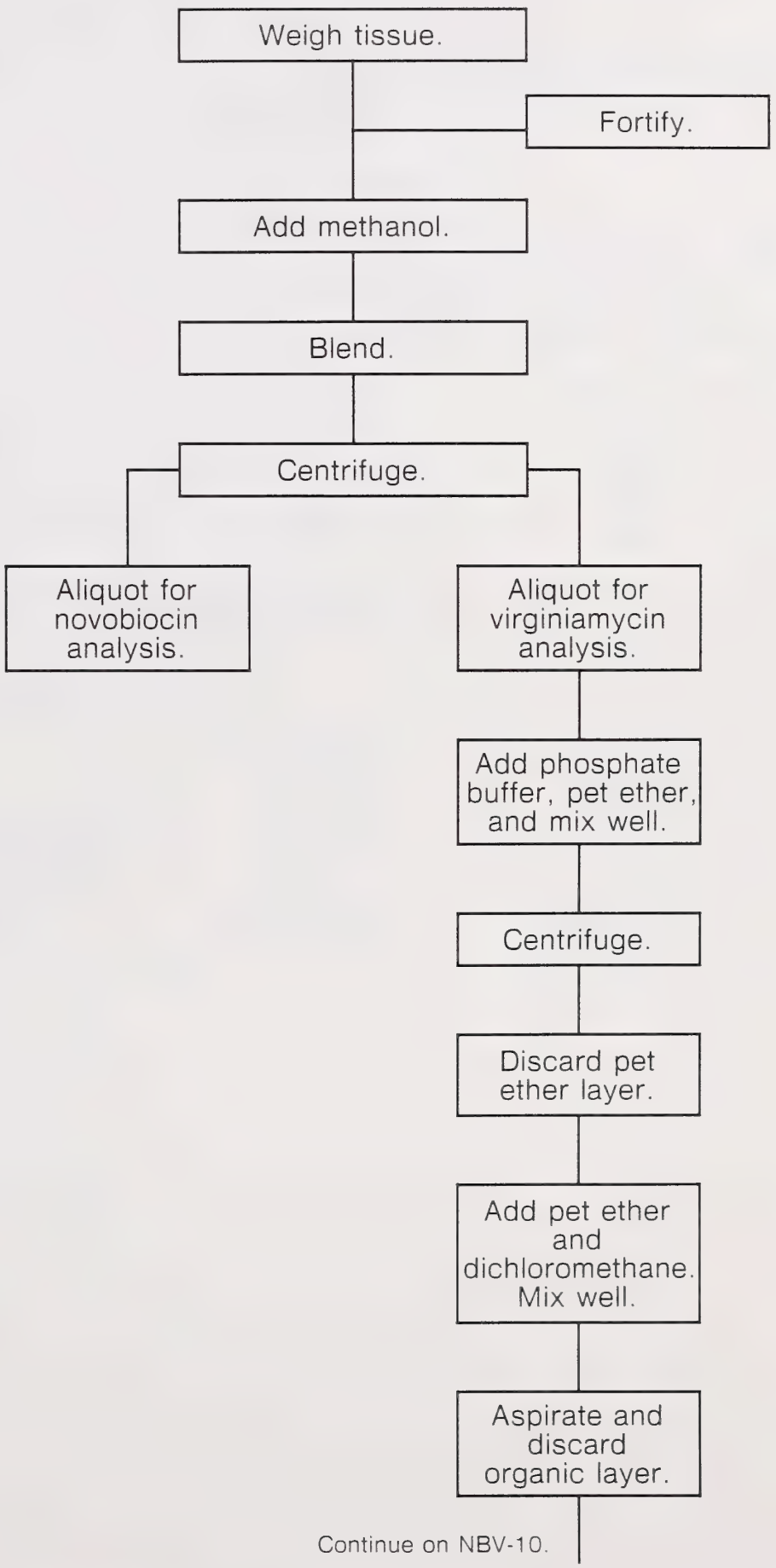
c. Inject 25 μL of the appropriate high level HPLC standard (0.666 μg/mL for novobiocin and 1.6 μg/mL for virginiamycin).

d. Inject 25 mL of each tentatively positive sample.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

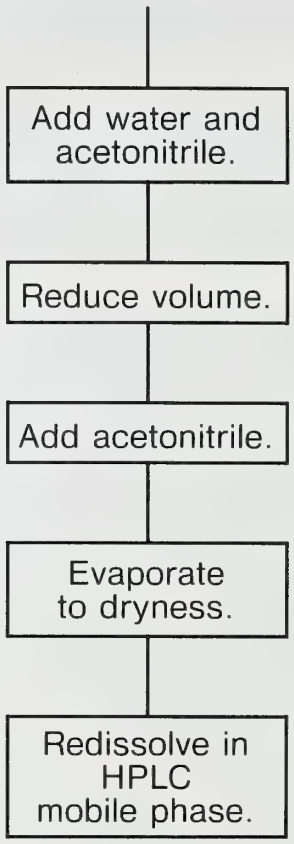
4. Flow Chart Summary



Continue on NBV-10.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

Instrumental Settings and Conditions

HPLC conditions.

a. Column: Supelco LC-18 DB Column, 15 cm length, 4.6 mm i.d., 5 μm particle size.

b. Flow rate: 1.0 mL/min

c. Elution system: A gradient analysis is used in order to analyze both novobiocin and virginiamycin in a single injection. The gradient profile is as follows:

Time	*Solvent A(%)*	*Solvent B(%)*
0.2	94.0	6.0
30.0	15.0	85.0
35.0	15.0	85.0
40.0	94.0	6.0
45.0	94.0	6.0

Where Solvent A = 0.01M phosphoric acid
Solvent B = acetonitrile

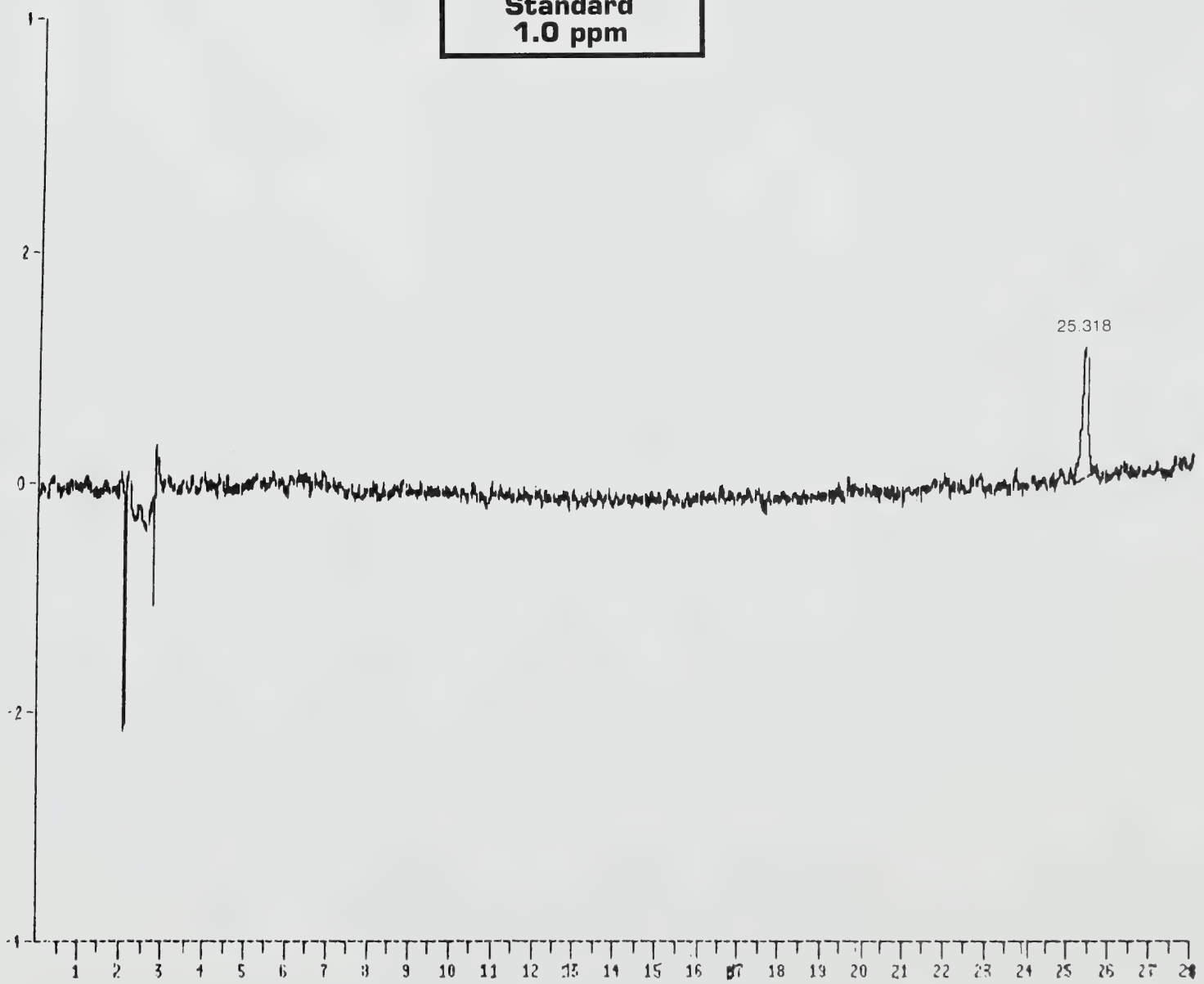
d. Diode array detector settings:

	Novobiocin	*Virginiamycin*
Wavelength	340 nm	230 nm
Bandwith	10 nm	10 nm
Reference wavelength	550 nm	550 nm
Reference bandwith	10 nm	10 nm
Sampling interval	640 ms	640 ms
Spectrum range	210-400 nm	210-400 nm

DETERMINATIVE METHOD

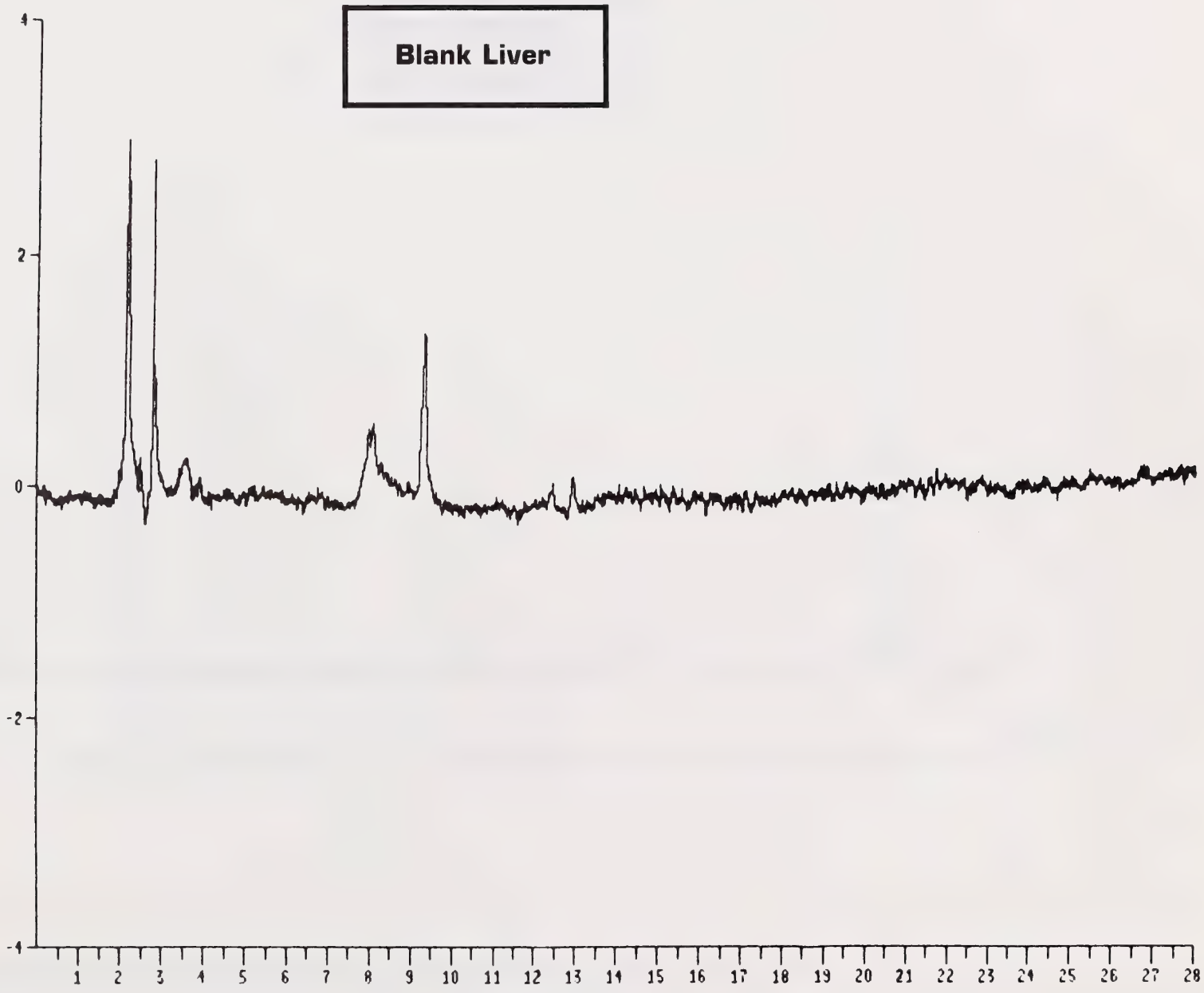
F. ANALYTICAL QUANTITATION (Continued)

Novobiocin Standard 1.0 ppm



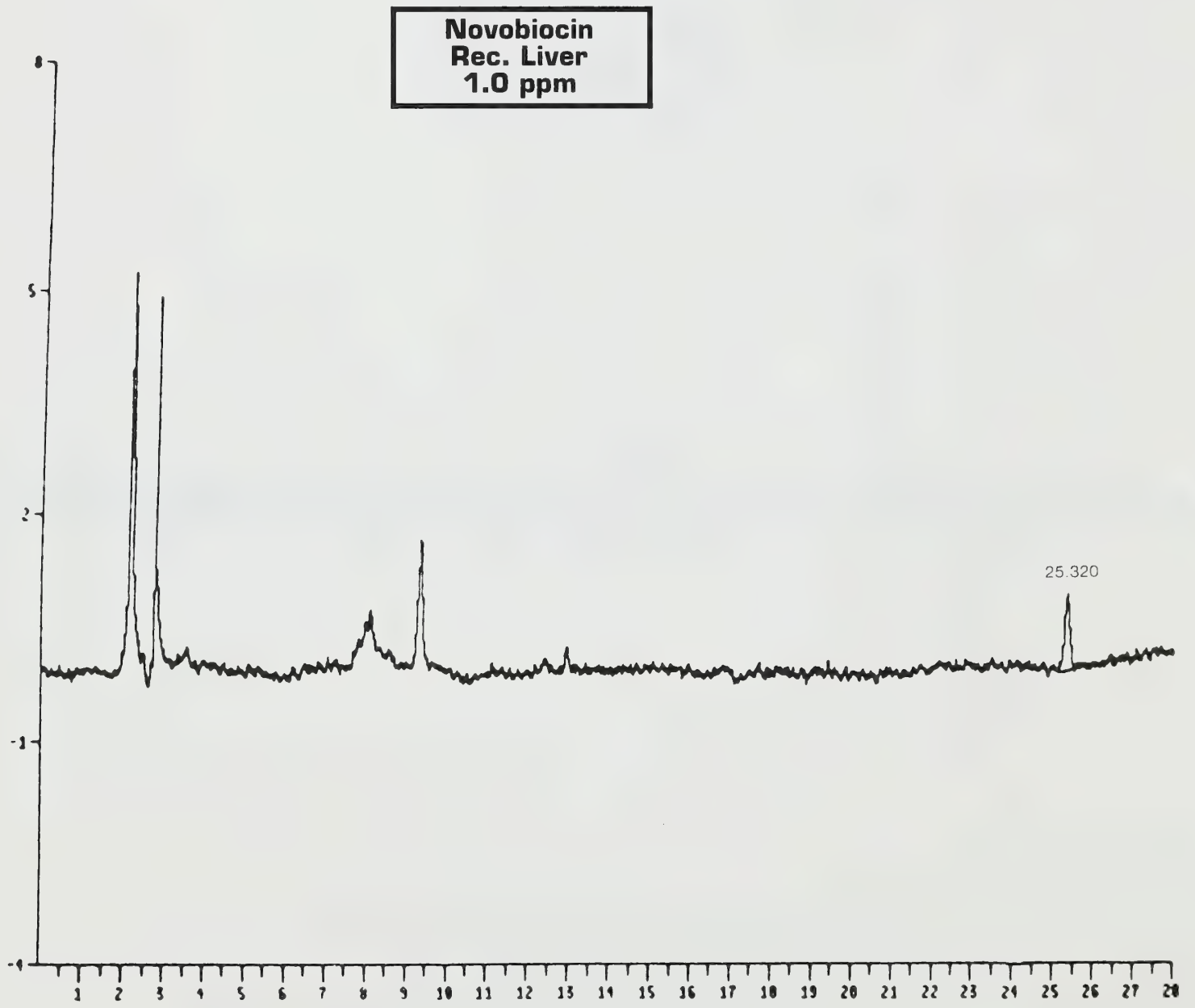
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

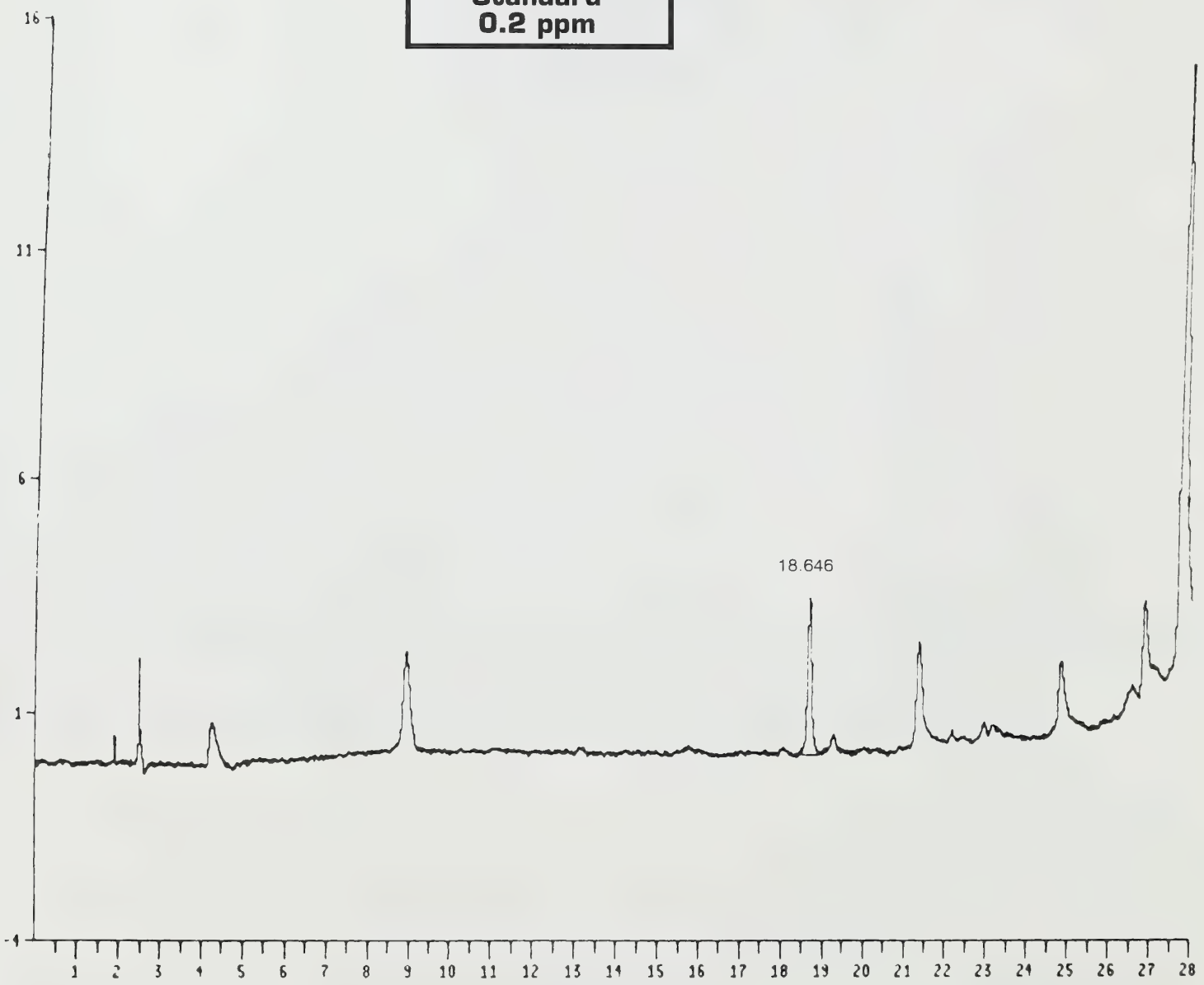
Novobiocin
Rec. Liver
2.0 ppm



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

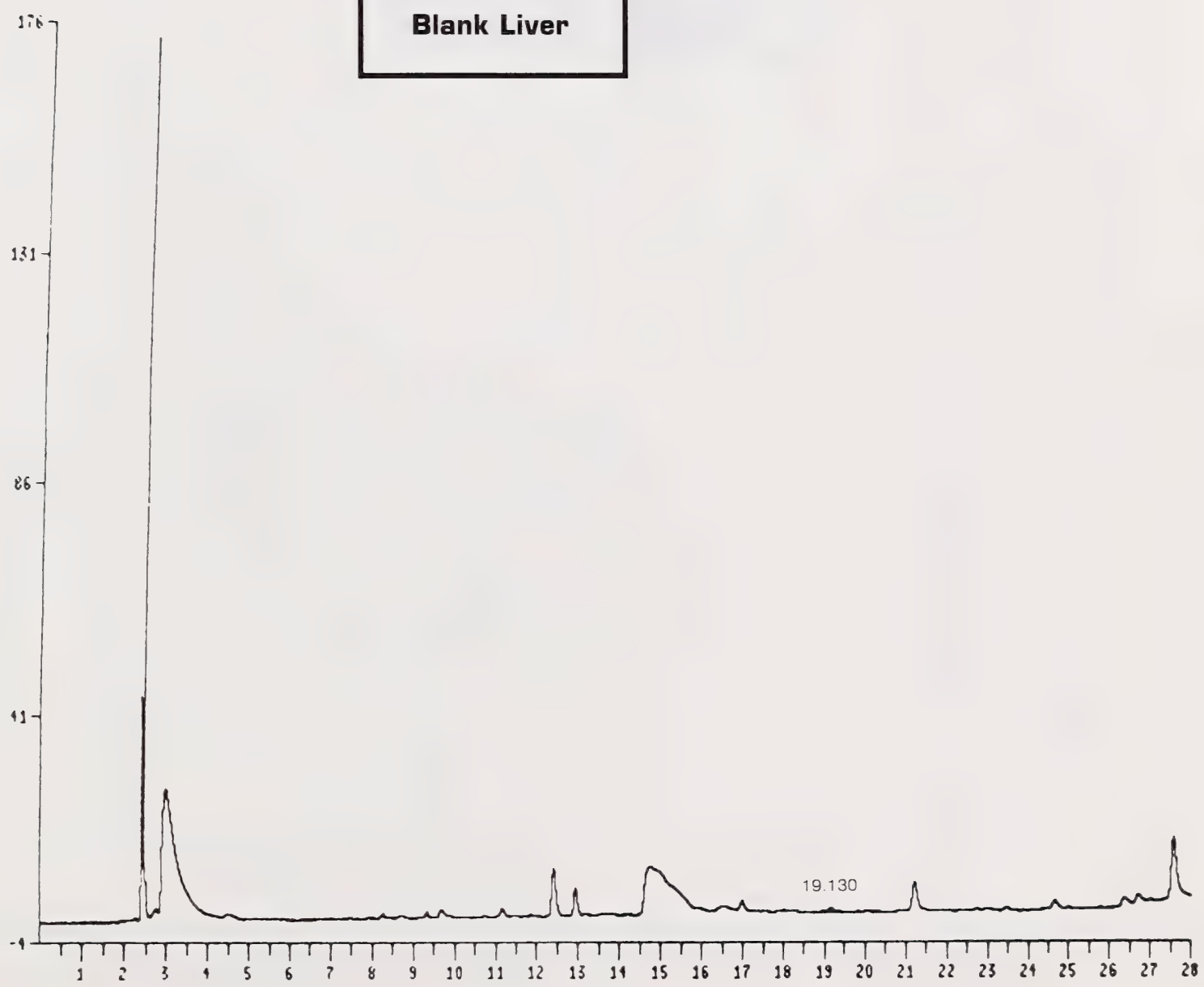
Virginiamycin Standard 0.2 ppm



DETERMINATIVE METHOD

F. ANALYTICAL QUANTIATION (Continued)

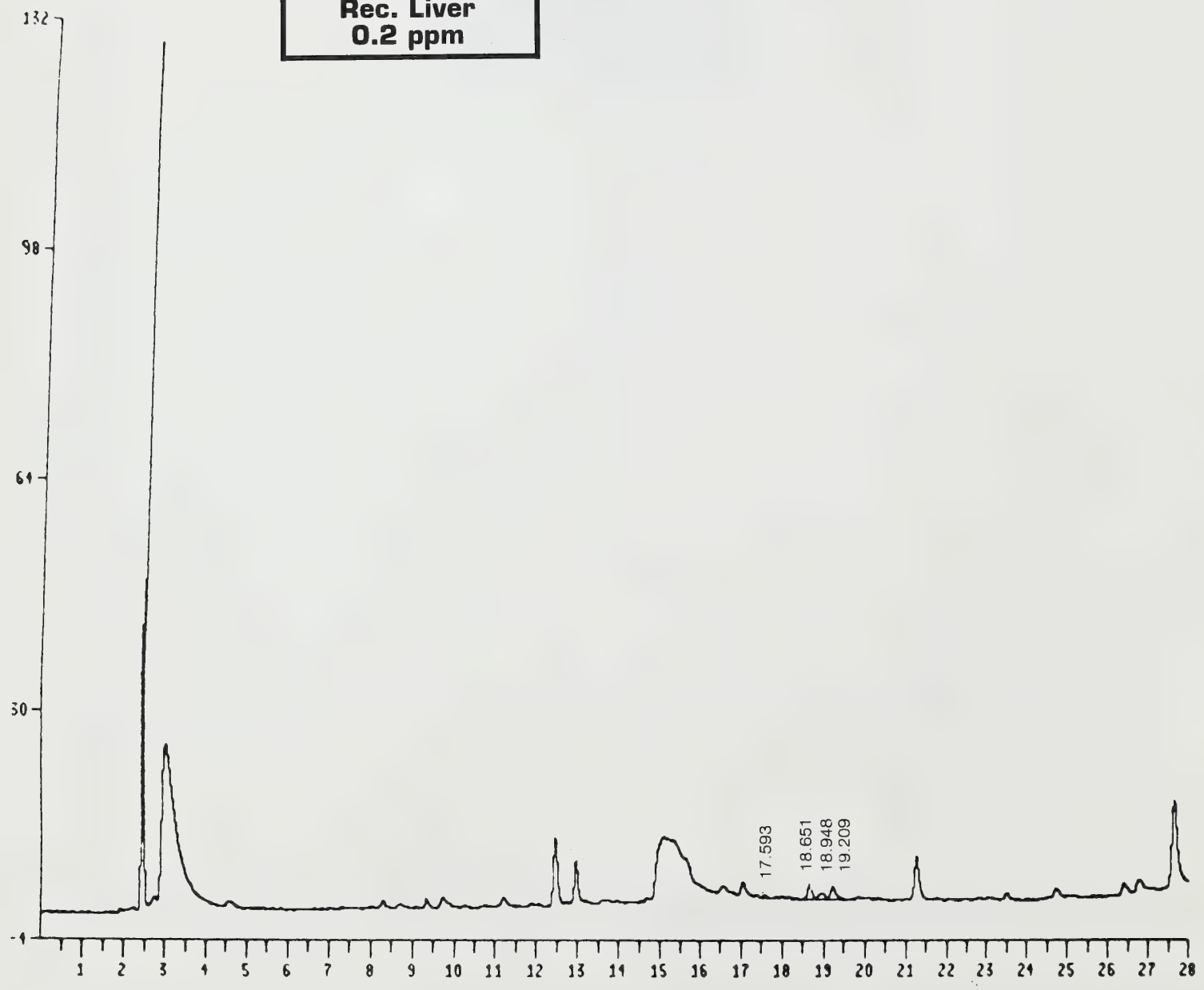
Blank Liver



DETERMINATIVE METHOD

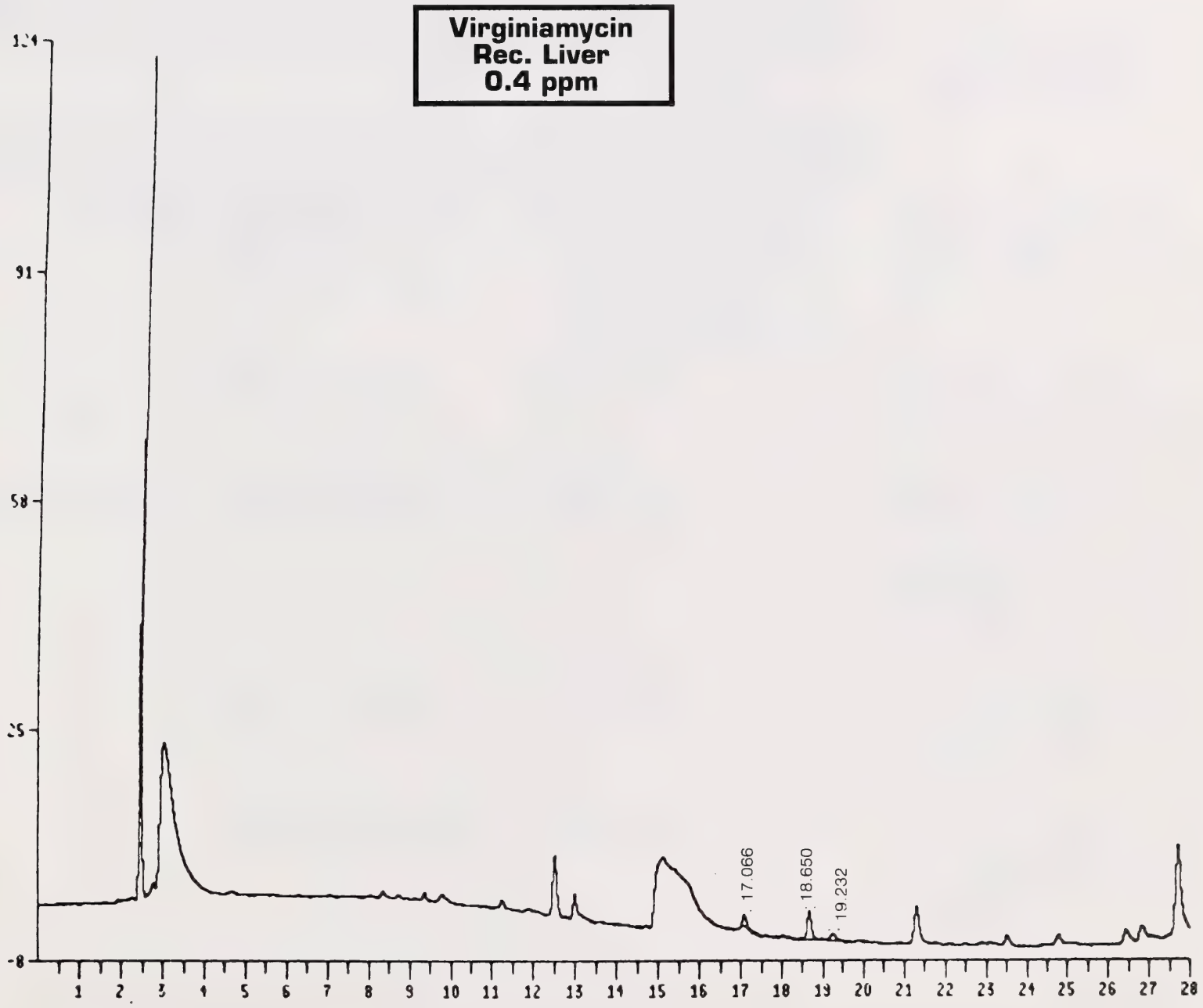
F. ANALYTICAL QUANTITATION (Continued)

Virginiamycin
Rec. Liver
0.2 ppm



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



DETERMINATIVE METHOD

G. CALCULATIONS

Procedure	By an acceptable means, measure the peak heights of the 0, 0.5, 1.0, and 2.0 ppm novobiocin standards. By an acceptable means, measure the peak heights of the 0, 0.1, 0.2, and 0.4 ppm virginiamycin standards. Using peak height and associated ppm values, construct a standard calibration curve by least squares calibration, as indicated in the Chemistry Quality Assurance Handbook Volume I, page 1.5.67. Using the standard curve, determine the concentration of novobiocin or virginiamycin in the sample. Determine percent recovery for the fortification standard. Use the 10-day running average to correct for recovery. The 10-day running average should be determined for each species/matrix combination. After it can be shown that there is no species/matrix bias on recoveries, the 10-day running averages do not have to be maintained for each species/matrix combination.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Analysis of Novobiocin and Virginiamycin in Animal Tissues

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Acetonitrile Petroleum ether Methanol Methylene chloride	These solvents are all flammable and may produce toxic effects to the skin, eyes, and respiratory system. Vapors are hazardous.	Work in efficient fume hood, away from electric heaters. Use plastic gloves.
Phosporic acid	Corrosive.	Wear eye protection, lab coat, and protective gloves.

4. Disposal Procedures

Organic solvents	See above	Store in organic solvent disposal container until disposed of by a contractor or in-house specialist.
Acidic solutions	See above	Dilute and flush into an acid disposal sink located in a well-ventilated area.
Methylene chloride	See above	Store with waste chlorinated solvents until disposed of by a contractor or in-house specialist.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Novobiocin and Virginiamycin*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

NOVOBIOCIN AND VIRGINIAMYCIN

SAMPLE NUMBER:

TISSUE:

ANALYST:

DATE:

Sample	*Retention Time*	*Peak Height/Area* *Novobiocin 340nm*	*Virginiamycin 230 nm*
Blank			
Standard			

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
Novobiocin	0-10.0	60-100	≤ 10.0	≤ 15.0
Virginiamycin	0- 4.0	60-100	≤ 10.0	≤ 15.0

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight	5.0 g ± 0.1 g
Volume of extraction solvent	15.0 mL ± 0.2 mL

3. Readiness To Perform

a. Familiarization

i. Novobiocin.

(a) Phase I: Duplicate sets of standard curves on each of three days at 0, 0.5, 1.0, and 2.0 ppm.

(b) Phase II: Self-fortified samples using beef liver tissue spiked at 0, 0.5, 1.0, and 2.0 ppm (duplicates) on three successive days.

NOTE: Phase I and Phase II may be performed concurrently.

(c) Phase III: Check samples for analyst accreditation.

(1) Twelve samples submitted by laboratory LSO.

(2) Report findings through LSO to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

ii. Virginiamycin.

(a) Phase I: Duplicate sets of standard curves on each of three days at 0, 0.1, 0.2, and 0.4 ppm.

(b) Phase II: Self-fortified samples using beef liver tissue spiked at 0, 0.1, 0.2, and 0.4 (duplicates) on three successive days.

NOTE: Phase I and Phase II may be performed concurrently.

(c) Phase III: Check samples for analyst accreditation.

(1) Twelve samples submitted by laboratory LSO.

(2) Report findings through LSO to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst. Blind samples or random duplicates chosen by the supervisor or LSO.

ii. Records to be maintained by analyst and reviewed by supervisor and LSO.

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

(d) Running average, standard deviation, and CV for all recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

(a) Stop all official analyses for that analyst.

(b) Investigate and identify probable cause.

(c) Take corrective action.

(d) Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver, muscle, or kidney.

b. Sample receipt size: Sufficient for all quantitative and confirmation analyses and sample reserve (500 g).

c. Condition upon receipt: Chilled or frozen.

d. Sample storage:

i. Time: 3 months.

ii. Condition: Store frozen at $-4°$ C or lower until analyzed.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	a. Blank. b. Fortification standard: 1.0 ppm novobiocin; 0.2 ppm virginiamycin. c. Samples.
7. Sensitivity	a. Lowest detectable level (LDL). i. Novobiocin: 0.5 ppm. ii. Virginiamycin: 0.1 ppm. b. Lowest reliable quantitation (LRQ). i. Novobiocin: 1.0 ppm. ii. Virginiamycin: 0.2 ppm. c. Minimum proficiency level (MPL). i. Novobiocin: 1.0 ppm. ii. Virginiamycin: 0.2 ppm.

ANALYSIS OF NOVOBIOCIN RESIDUES IN ANIMAL TISSUES USING ZYMATE ROBOTIC SYSTEM

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 8
G. Calculations 13
H. Hazard Analysis 14
I. Worksheet 15
J. Quality Assurance Plan 19

DETERMINATIVE METHOD

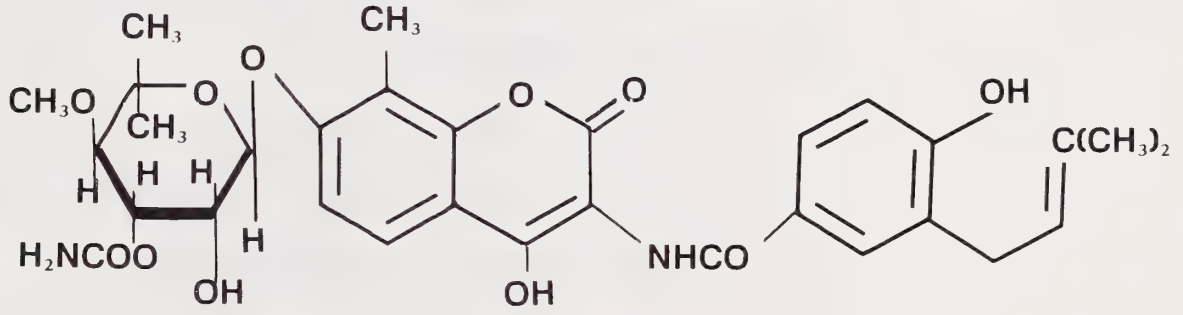
A. INTRODUCTION

1. Theory and Structure

The antibiotic compound novobiocin (NOV) is used in the treatment of meat-producing animals. Primary control of antibiotic residues in the domestic food supply is accomplished using microbiological assay methodology. This method was developed to provide chemical identification of novobiocin in tissues. Additionally, the method provides quantitative supporting data for the official biological method.

Novobiocin residue is extracted from tissue with methanol. The homogenate is centrifuged to separate solid materials, and an aliquot of the clear methanol layer is used for analysis. Separation and detection is accomplished using a C_{18} reversed phase radial compression column and UV detection at 340 nm.

The method has been automated, using a Zymate Pytechnology Laboratory Robotic System. Using this system, one analyst may prepare 24 samples per day with approximately one hour of analyst set-up time. Prepared samples are manually transferred to HPLC autosampler vials for analysis.



2. Applicability

This method is applicable to bovine, porcine, and avian species, and liver, muscle, or kidney tissue.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. 50 mL polypropylene centrifuge tubes, Falcon Blue Max, #2070, or equivalent.

b. 16 × 100 mm culture tubes, Scientific Products T1290-6, or equivalent.

c. 5 mL pipet tips, Oxford Type #814, or equivalent.

2. Instrumentation

a. Liquid chromatographic system.

i. Varian Model 210 HPLC pump.

ii. Water radial compression module.

iii. Waters C_{18} 10μm radial compression column.

iv. Spectra Physics Model 481 Lambada Max LC spectrophotometer.

v. WISP Model 710A automatic injection system.

b. Zymark robotic system.

i. ZP-100 Zymate II Core System.

ii. ZP-710 centrifuge station, 50 mL tubes, four-station capacity.

iii. ZP-013-7 50 mL centrifuge rack.

iv. Tissuemizer section (custom manufactured by Zymark, Inc.).

v. ZP-911-1 pipetting hand.

vi. ZP-900-2 general purpose gripping hand.

vii. ZP-510 Master Lab solvent dispensing station.

viii. Disposal station.

ix. ZP-830 power and event controller.

x. ZP-013-3 16 × 100 mm test tube rack.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Methanol, HPLC grade, MCB Omnisolv, or equivalent.

b. Phosphoric acid, 85%, Malinckrodt catalog # 2761-1, or equivalent.

c. Acetonitrile, HPLC grade, Burdick and Jackson, or equivalent.

d. 0.01M phosphoric acid/acetonitrile, 1:1 (v/v).

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Novobiocin: Sigma Chemical catalog #N-1628.

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178

2. Preparation of Standards

a. Stock standard solutions (50 μg/mL).

Accurately weigh 5.0 mg $\pm$ 0.1 mg novobiocin reference standard material into a clean 100 mL volumetric flask. Dissolve and dilute to volume with HPLC grade methanol.

b. Fortification standards.

Stock standard solution will be used as described in section E.2.

c. HPLC reference standards.

i. Pipet 20 mL of novobiocin stock standard solution into a clean 100 mL volumetric flask and dilute to volume with HPLC grade methanol. Concentration is 10 μg/mL.

ii. Pipet 1.66 mL of novobiocin solution c.i into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.166 μg/mL, equivalent to 0.5 ppm tissue concentration.

iii. Pipet 3.33 mL of novobiocin solution c.i into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.333 μg/mL, equivalent to 1.0 ppm tissue concentration.

iv. Pipet 6.66 mL of novobiocin solution c.i into a clean 100 mL volumetric flask and dilute to volume with methanol. Concentration is 0.666 μg/mL, equivalent to 2.0 ppm tissue concentration.

3. Storage Conditions

Stock solutions should be stored tightly stoppered at 4° C.

4. Shelf Life Stability

Stock solutions are stable up to 6 months if stored as described above.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 5.0 g ± 0.1 g of homogenized tissue into a disposable 50 mL centrifuge tube.

Fortification—Recovery sample is fortified using the novobiocin stock standard solution as follows: 1.0 ppm tissue level—add 100 μL of novobiocin stock solution.

b. Add 15.0 mL methanol.

c. Blend for 60 sec at medium speed using a Polytron tissuemizer.

d. Centrifuge for 5 min at 2000 rpm. Transfer clear organic layer to a clean 50 mL polypropylene centrifuge tube.

e. Allow extract to stand overnight in a refrigerator.

f. Inject 15 μL of the extract for liquid chromatographic analysis.

2. Screening Analysis

Since most samples analyzed by this method will be negative, it is more efficient to screen all samples before performing the injections necessary for a quantitative determination. To perform the screening analysis the following steps are required:

a. Inject 15 μL of the 0.333 μg/mL (1.0 ppm tissue equivalent) novobiocin HPLC reference standard.

b. Inject 15 μL of each sample extract and fortification standard.

c. Evaluate sample extracts for a tentative positive finding. A tentative is identified if both the following conditions are true:

   i. There is a peak within 0.2 min of the expected retention of novobiocin established from the standard injection in step 2.a above.

   ii. The identified peak is ≥ 20% of the peak height established for novobiocin by the standard injection.

d. If both of these conditions are met, the sample is identified as ''tentatively'' positive. Proceed with the quantitative part of this determination below.

3. Quantitative Determination

a. Inject 15 μL of each novobiocin HPLC reference standard solution.

b. Inject 15 μL of each sample that has been identified as tentatively positive.

c. Inject 15 μL of the recovery sample.

The above analysis is completely described by the program NOVO, which was written for the Zymate Pytechnology robotic system. This program is shown in E.4.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

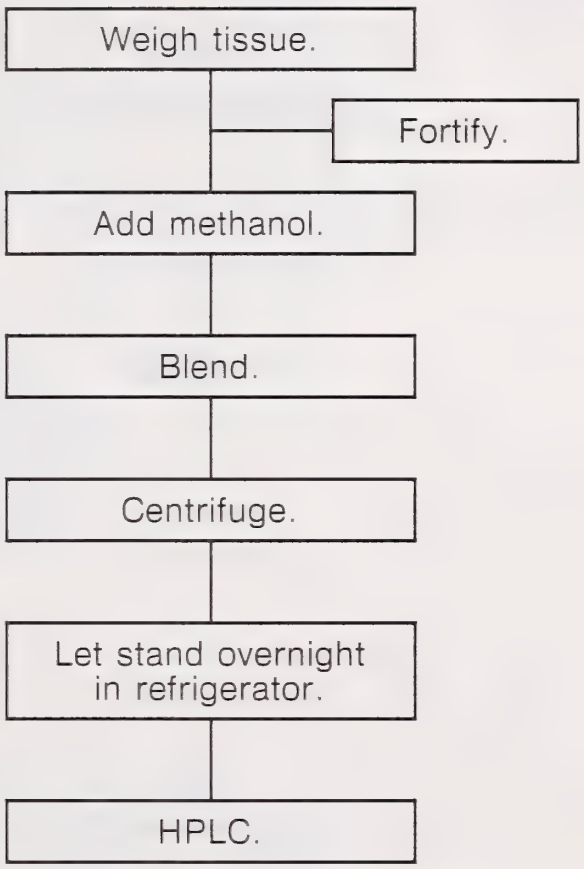
4. NOVO

```
      PROMPT How many samples in this set?
      INPUT TOTAL.SAMPLES
      SET.INITIAL.CONDITIONS
      SAMPLE.NUMBER = 1
 20   RACK.7.INDEX = SAMPLE.NUMBER
      RACK.2.INDEX = SAMPLE.NUMBER
      GET.A.SAMPLE
      HOMOGENIZE.SAMPLE
      S:VOL = S:VOL + 15
 40   CENTRIFUGE.INDEX = 1
      PUT.TUBE.IN.CENTRIFUGE
      TIMER(6) = 3000
      WASH.TISSUEMISER
 80   WAIT FOR TIMER 6
      GET.TUBE.FROM.CENTRIFUGE
100   PUT.TUBE.IN.RACK.7
110   GET.SML.TIP
      PIPET.VOLUME = 3.0
      MOVE.OVER.RACK.7
      ASPIRATE.SML.TIP
      RACK.2.INDEX = SAMPLE.NUMBER
      MOVE.OVER.RACK.2
      DISPENSE.SML.TIP
      DISPOSE.TO.WASTE
      SAMPLE.NUMBER = SAMPLE.NUMBER + 1
      IF SAMPLE.NUMBER > TOTAL.SAMPLES THEN GOTO 1000
      GOTO 20
1000  PARK.HAND$
```

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

5. Flow Chart Summary



DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

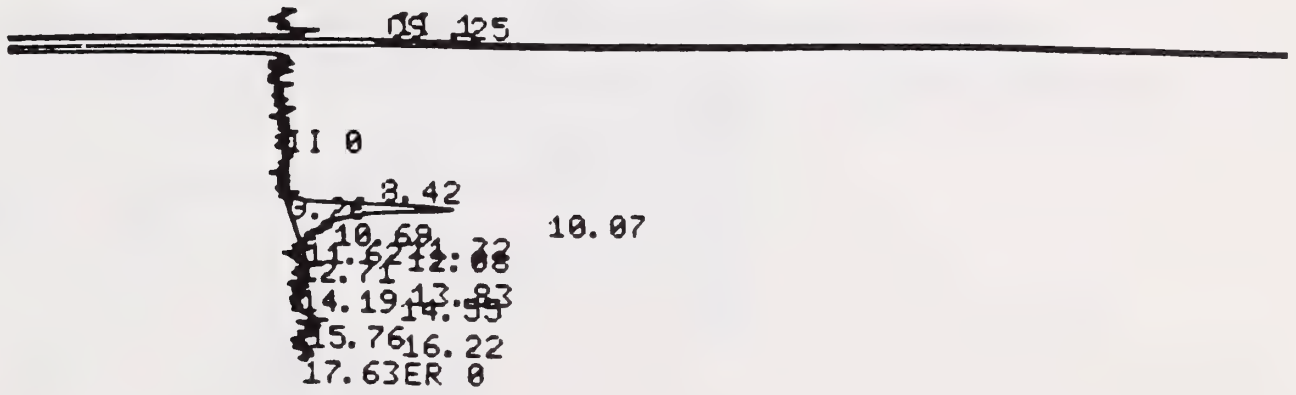
1. Instrumental Settings and Conditions	a. Column	C_{18} 10μm radial compression column
	b. Mobile phase	0.01M phosphoric acid/acetonitrile (1:1)
	c. Flow rate	1.0 mL/min
	d. Detector wavelength	340 nm
	e. Detector sensitivity	0.01 AFS (or suitable scale for concentration range)

DETERMINATIVE METHOD

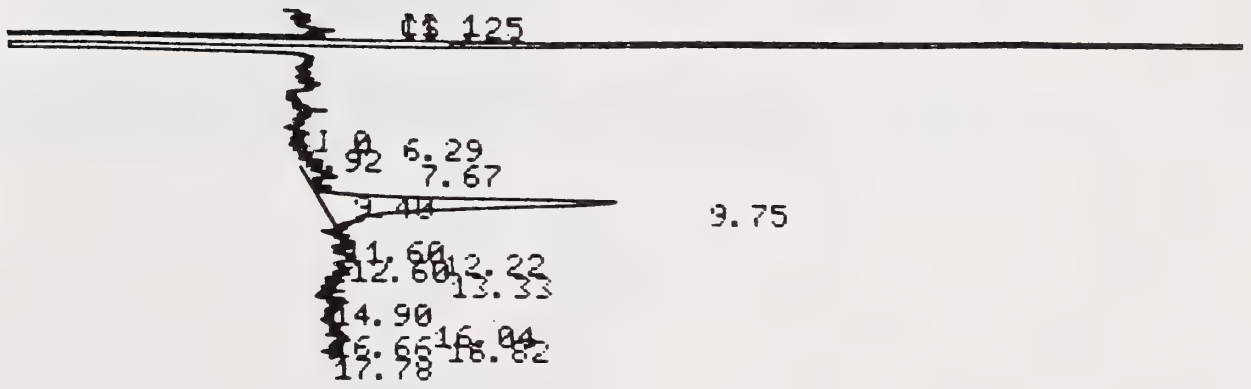
F. ANALYTICAL QUANTITATION (Continued)

2. Sample Chromatograms

STD
0.5 ppm novobiocin



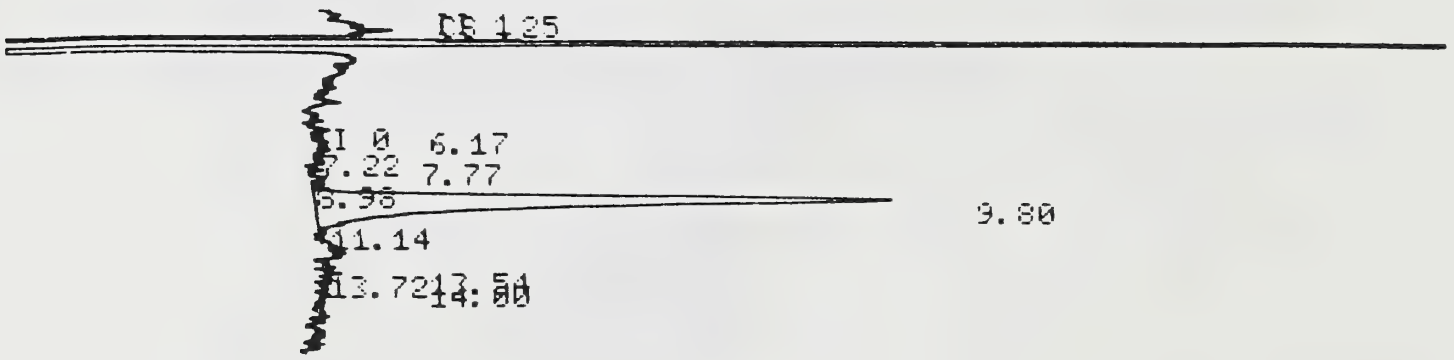
STD
1.0 ppm novobiocin



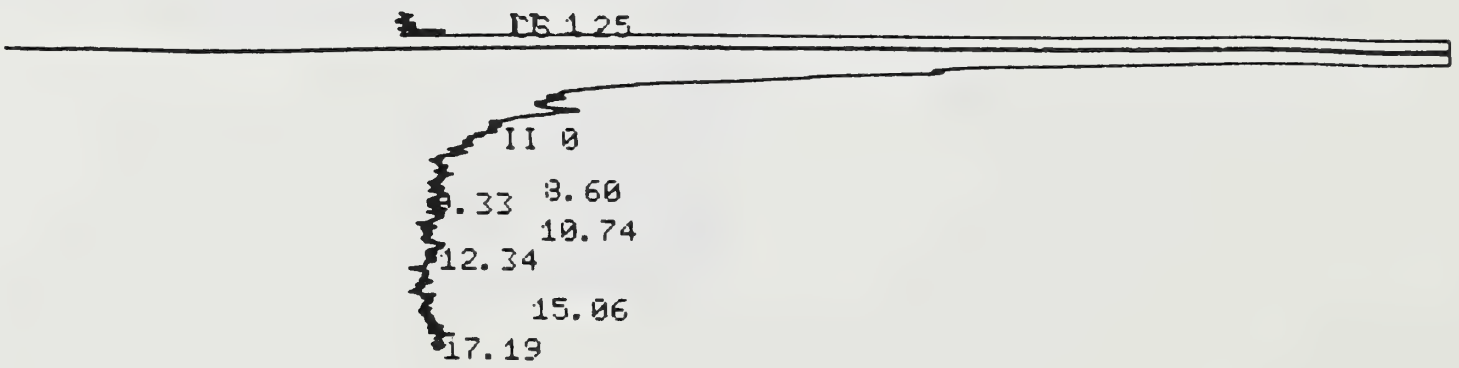
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

STD
2.0 ppm novobiocin



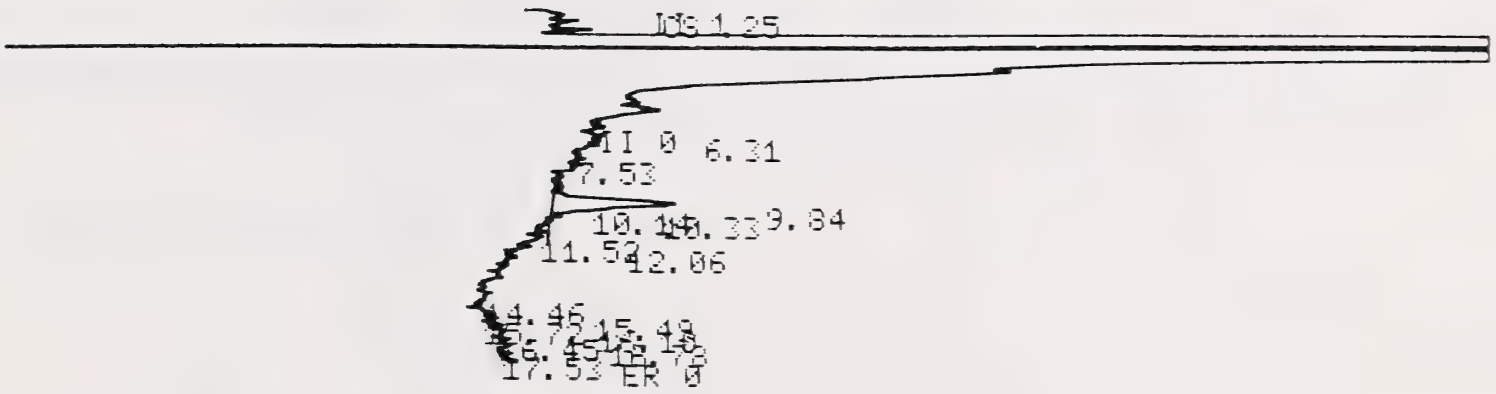
Blk
Beef Liver



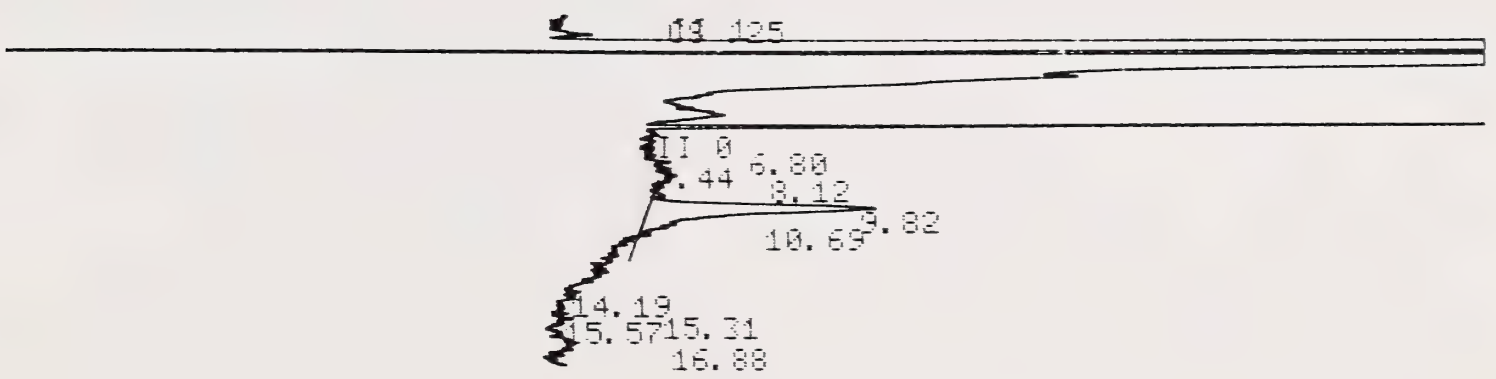
DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

Recovery
0.5 ppm Beef Liver



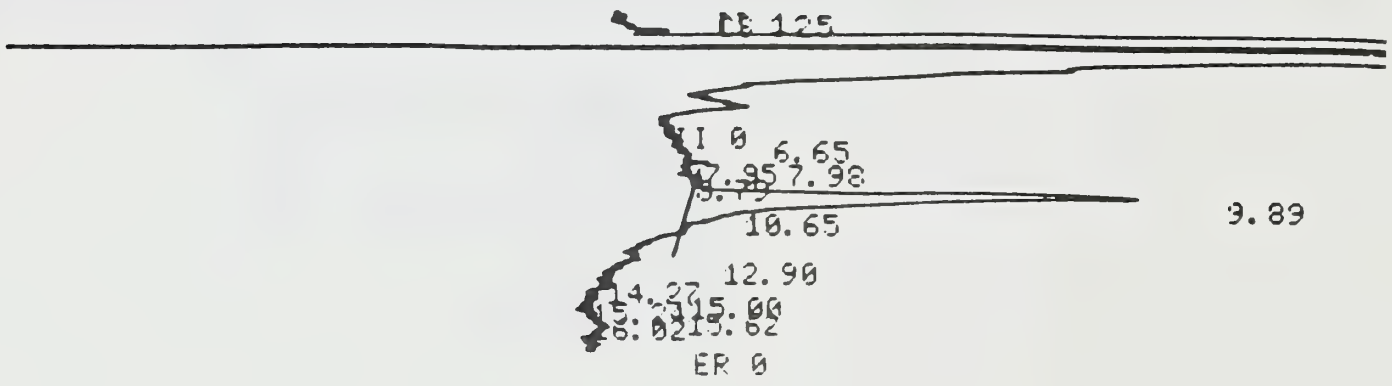
Recovery
1.0 ppm Beef Liver



DETERMINATIVE METHOD

F. ANALYTICAL QUANTIATION (Continued)

Recovery
2.0 ppm Beef Liver



DETERMINATIVE METHOD

G. CALCULATIONS

Procedure	By an acceptable means, measure the peak heights of the 0, 0.5, 1.0, and 2.0 ppm novobiocin standards. Using peak height and associated ppm values, construct a standard calibration curve by least squares calibration, as indicated in the Chemistry Quality Assurance Handbook, Vol. I, page 1.5.67. Using the standard curve, determine the concentration of novobiocin in the sample. Determine percent recovery for the fortification standard. Use the 10-day running average to correct for recovery. The 10-day running average should be determined for each species/matrix combination. After it can be shown that there is no species/matrix bias on recoveries, 10-day running averages do not have to be maintained for each species/matrix combination.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Novobiocin in Animal Tissues Using Zymate Robotic System		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Acetonitrile Methanol	These solvents are all flammable and may produce toxic effects to the skin, eyes, and respiratory system. Vapors are hazardous.	Work in efficient fume hood, away from electric heaters. Use plastic gloves.
	Phosphoric acid	Corrosive.	Wear eye protection, lab coat, and protective gloves.
4. Disposal Procedures	Acidic solutions	See above	Dilute and flush into an acid disposal sink located in a well-ventilated area.
	Organic solvents	See above	Store in organic solvent disposal container until disposed of by a contractor or in-house specialist.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Novobiocin*, can be removed from this book from photocopying whenever necessary, but do not forget to replace it.

NOVOBIOCIN

Sample Number:
Tissue:
Analyst:
Date:

	Retention Time	Peak Height or Area
Blank		
Standard		

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
Novobiocin	0-10.0	60-100	≤ 10.0	≤ 15.0

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	5.0 g ± 0.1 g
b. Volume of extraction solvent	15.0 mL ± 0.2 mL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Duplicate sets of standard curves on each of three days at 0, 0.5, 1.0, and 2.0 ppm.

ii. Phase II; Self-fortified samples using beef liver tissue spiked at 0, 0.5, 1.0, and 2.0 (duplicates) on three successive days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Twelve samples submitted by laboratory LSO.

(b) Report analytical findings through LSO to Chemistry Division.

Written approval from Chemistry Division required to commence official analysis.

b. Acceptable criteria.

i. Refer to section J.1 above.

ii. No false positive or false negative findings.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst. Blind samples or random duplicates chosen by the supervisor or LSO.

ii. Records to be maintained by analyst and reviewed by supervisor and LSO.

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

(d) Running average, standard deviation, and CV for all recoveries.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

i. CV equal to or better than that stated in section J.1.

ii. CV for running average of the percent recoveries < 10%, with all recoveries to range as stated in section J.1.

iii. If unacceptable values are obtained, then:

(a) Stop all official analyses for that analyst.

(b) Investigate and identify probable cause.

(c) Take corrective action.

(d) Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Bovine, porcine, or avian liver, muscle, or kidney.

b. Sample receipt size: Sufficient for all quantitative and confirmation analyses and sample reserve (500 g).

c. Condition upon receipt: Chilled or frozen.

d. Sample storage:

i. Time: 3 months.

ii. Condition: Store frozen at −4° C or lower until analyzed.

6. Sample Set

a. Blank.

b. Fortification standard (1.0 ppm novobiocin).

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 0.25 ppm.

b. Lowest reliable quantitation (LRQ): 1.0 ppm.

c. Minimum proficiency level (MPL): 0.5 ppm.

NICARBAZIN IN POULTRY TISSUES

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 9
G. Calculations 14
H. Hazard Analysis 15
I. Worksheet 16
J. Quality Assurance Plan 19

II. Confirmatory Method

A. Introduction 21
B. Equipment 21
C. Reagents and Solutions 21
D. Standards 21
E. Sample Preparation and Cleanup 22
F. Analytical Confirmation Procedure 22
G. [Reserved]
H. Hazard Analysis 24
I. Worksheet 24
J. Quality Assurance Plan 27

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Nicarbazin is a 1:1 molar mixture of HDP and DNC. HDP is 4,6-Dimethyl-2-pyrimidinol and DNC is 4,4-Dinitrocarbanilide. Nicarbazin is used in chickens to prevent coccidiosis, an infectious disease caused by intestinal protozoan parasites.

Nicarbazin is extracted from chicken tissue with ethyl acetate. After filtration and evaporation of the extract, the residue is purified by liquid-liquid partitioning and alumina column chromatography. Analytical separation and measurement are accomplished by high performance liquid chromatography (HPLC) with UV detection of the DNC portion of the nicarbazin complex at 340 nm.

2. Applicability

This method is applicable for chicken muscle and liver.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Equivalent apparatus may be substituted for that specified if necessary.

a. Platform shaker, Eberbach Model 6010.

b. Cieria homogenizer, Sorvall Omni-Mixer, Type OM. NOTE: A Tissuemizer or equivalent *cannot* be used.

c. Virtis flask (100 mL).

d. Du Pont homogenizing cups, part no. 17047.

e. Rinco rotary vacuum evaporator with temperature-controlled water bath.

f. Waters Sep-Pak alumina B cartridges, part no. 51820.

g. Filter paper, Schleicher and Schuell 588.

h. 50 mL polypropylene tubes, Falcon 2098.

i. Waring blender, with 1 quart jar.

j. Hobart food chopper, equipped with 5/32″ hole plate.

2. Instrumentation

HPLC system consisting of the following components, or equivalent:

a. Waters Model M-6000 high pressure pump.

b. Waters Wisp Model 710B autosampler and autoinjector.

c. Waters Model 450 UV-visible detector, set at 340 nm.

d. Chromatographic data system, HP 1000, integrator, or chart recorder.

e. IBM C_{18} column, 4.6 × 100 mm, 3 μm particles.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Ethyl acetate, HPLC grade.

b. Hexane, HPLC grade.

c. Acetonitrile, HPLC grade.

d. Dimethylformamide (DMF), HPLC grade.

e. Methanol, HPLC grade.

f. Water, deionized using Millipore system.

2. Solution List

Methanol/water, 3: 1 (v/v).

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Eli Lilly and Company
Indianapolis, IN 46285

NOTE: Each new lot of standard reference material should be prepared as stated below and compared to the current standard.

NOTE: Information as to the exact ratio of DNC to HDP for that lot should be provided and will be needed for calculations.

2. Preparation of Standards

a. Stock standard (1000 μg/mL): Weigh 100 mg of nicarbazin reference standard. Transfer to a 100 mL volumetric flask and dissolve in 75 mL of dimethylformamide (DMF) by heating at 75-80° C. Cool, dilute to volume with DMF, and mix well.

b. Working standard (20 μg/mL): Pipet 2.0 mL of stock standard solution into a clean 100 mL volumetric flask. Dilute to volume with ethyl acetate.

c. HPLC standards:

i. 6 μg/mL spiking solution. Pipet 3.0 mL of working standard into a 10 mL volumetric flask and dilute to volume with methanol.

ii. 4 μg/mL spiking solution. Pipet 2.0 mL of working standard into a 10 mL volumetric flask and dilute to volume with methanol.

iii. 2 μg/mL spiking solution. Pipet 1.0 mL of working standard into a 10 mL volumetric flask and dilute to volume with methanol.

3. Storage Conditions

All standard solutions should be stored at room temperature.

4. Shelf Life Stability

a. Stock standard—stable for one month.

b. Working standard—stable for one month.

c. HPLC standard—stable for three days.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

a. Liver. Blend approximately 1 lb of tissue in a 1-qt Waring blender jar until a consistent homogenate is obtained.

b. Muscle. Pass approximately 1 lb of tissue through a Hobart food chopper equipped with a 5/32″ hole plate. Mix thoroughly by hand-kneading to produce a uniform homogenate.

NOTE: *Do not* homogenize tissues completely. A Tissuemizer or equivalent *cannot* be used since the homogenate produces interferences in recovery of analyte and filters slowly as a consequence of high efficiency blending.

2. Sample Extraction

a. If using:

i. Eberbach platform shaker: Weigh 10.0 g of a representative sample of ground or minced tissue into a 50 mL polypropylene centrifuge tube.

ii. Virtis homogenizer: Weigh 10.0 g of a representative sample of ground or minced tissue into a 100 mL Virtis flask.

b. Prepare a recovery sample at 2 ppm by fortifying a 10.0 g sample of untreated control tissue with 1.0 mL of working standard. Include an untreated control tissue sample with each set of samples.

c. If using:

i. Eberbach platform shaker: Add 25 mL of ethyl acetate to the sample and shake by hand vigorously for 10 sec. Place on the platform shaker and shake for 3 min at high speed. Centrifuge the sample at 2000 rpm for 2 min.

ii. Virtis homogenizer: Add 25 mL of ethyl acetate to the sample and homogenize at low speed for 3 min.

d. Being careful to avoid transferring tissue, decant the ethyl acetate supernatant from either step c.i or c.ii into a 250 mL round-bottom flask or other suitable container.

NOTE: When blending sample in a Virtis homogenizer, decant supernatant through a filter paper. Any tissue that is decanted with the supernatant should be scraped off the filter paper and placed back into the Virtis flask.

e. Repeat steps c.i to d or c.ii to d twice more, using the same filter paper and flask to collect the extract from each sample. After the three extracts have been collected, rinse the filter paper with three 2 mL portions of ethyl acetate.

f. Evaporate the ethyl acetate using a rotary vacuum evaporator with a temperature-controlled water bath at 45-50° C.

NOTE: For liver and muscle tissue, there will be about 1-2 mL of oil left in the residue.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

g. Add 50 mL of hexane to the round-bottom flask and quantitatively transfer to a 125 mL separatory funnel.

h. Rinse the round-bottom flask with 10 mL acetonitrile and transfer to the separatory funnel. Shake the separatory funnel vigorously for approximately 1 min. Let the separatory funnel sit until the phases are sufficiently separated.

i. Carefully drain the acetonitrile layer (lower phase) into the same round-bottom flask previously used, ensuring that all of the acetonitrile layer is recovered.

j. Re-extract the hexane phase with two 10 mL portions of acetonitrile and add these acetonitrile extracts to the same round-bottom flask for each sample. Discard the hexane phase.

k. Evaporate the acetonitrile extract completely to dryness with a rotary vacuum evaporator. Reconstitute the dried extract by adding 2 mL of dimethylformamide to the flask.

l. Attach a 10 mL syringe barrel or similar apparatus to the alumina B cartridge as a solvent reservoir. Rinse the alumina B cartridge with 4 mL of DMF. *Do not allow the column to go dry at this step.*

NOTE: All elution steps are performed as an open column procedure without vacuum. It may be necessary to apply gentle pressure with a rubber bulb or other similar device to initiate flow.

m. Quantitatively transfer the solubilized sample to the alumina B cartridge by rinsing the sample flask with two 2 mL portions of DMF and by adding these rinses to the cartridge. Discard eluate.

n. Wash the cartridge with one 5 mL portion of DMF and discard eluate. It is important to eliminate any DMF with pressure from a rubber bulb.

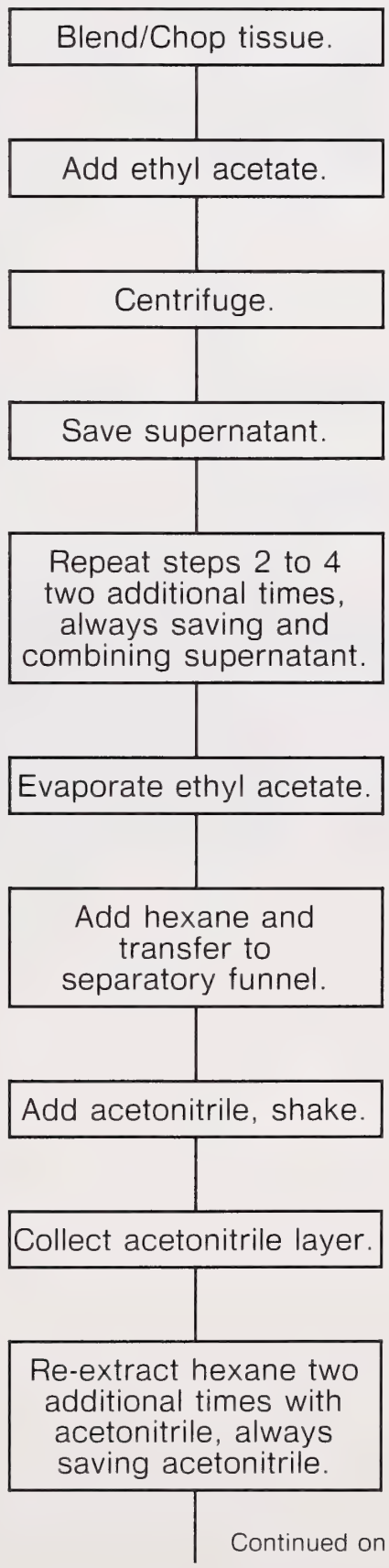
o. Elute the compound of interest into a 10 mL volumetric flask by adding 10 mL of methanol to the cartridge. A rubber bulb can be used to increase the flow rate to approximately 2 mL/min and to drain all of the DNC from the column. Adjust final volume in volumetric flask to mark, using methanol.

p. Inject 10 μL of sample into HPLC for quantitation.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

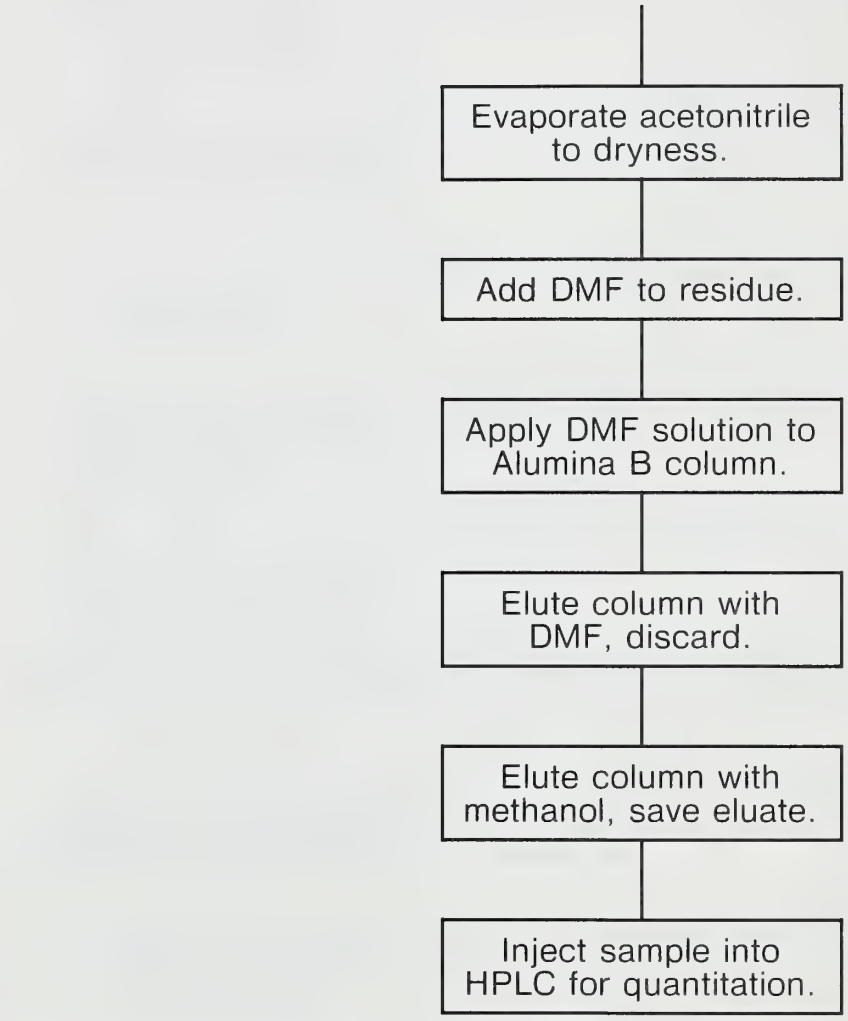
3. Flow Chart Summary



Continued on NIC-8

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. Guard column	C_{18}, 3 μm particles (optional)
b. Analytical column	IBM C_{18} column, 4.6 × 150 mm, 3 μm particles.
c. Mobile phase	Methanol/Water 75/25 (v/v).
d. Flow rate	0.8 mL/min.
e. Injection volume	10 μL.
f. Column temperature	40° C.
g. Retention time	Approximately 5-6 min.
h. Detector setting	UV 340 nm (0.1 AUFS for samples ≤ 0.5 ppm, and 0.2 AUFS for samples > 0.5 ppm).

2. General Operation

a. The baseline should be stable before beginning a run. Reference standard responses should be sufficient for reliable measurement (> 2 × signal to noise ratio). DNC should be separated from impurities in the sample. The retention time of DNC should not vary more than 20 sec in a particular run.

b. Record the daily operating conditions on the chromatograms and measure the peak area of DNC. If necessary, dilute sample extract with mobile phase to yield peak responses within the range of the standard curve.

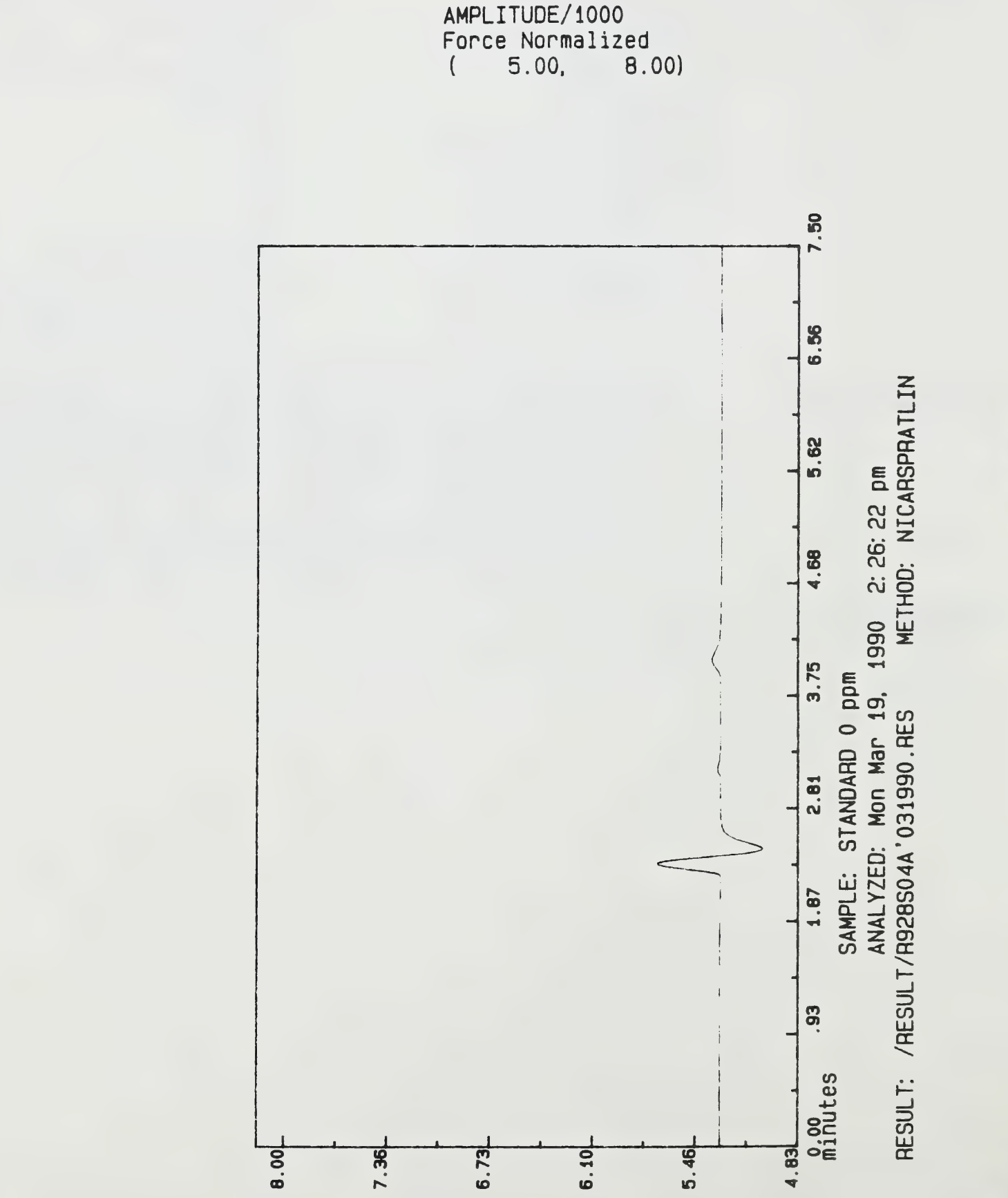
3. Interferences

None known.

I. DETERMINATIVE METHOD

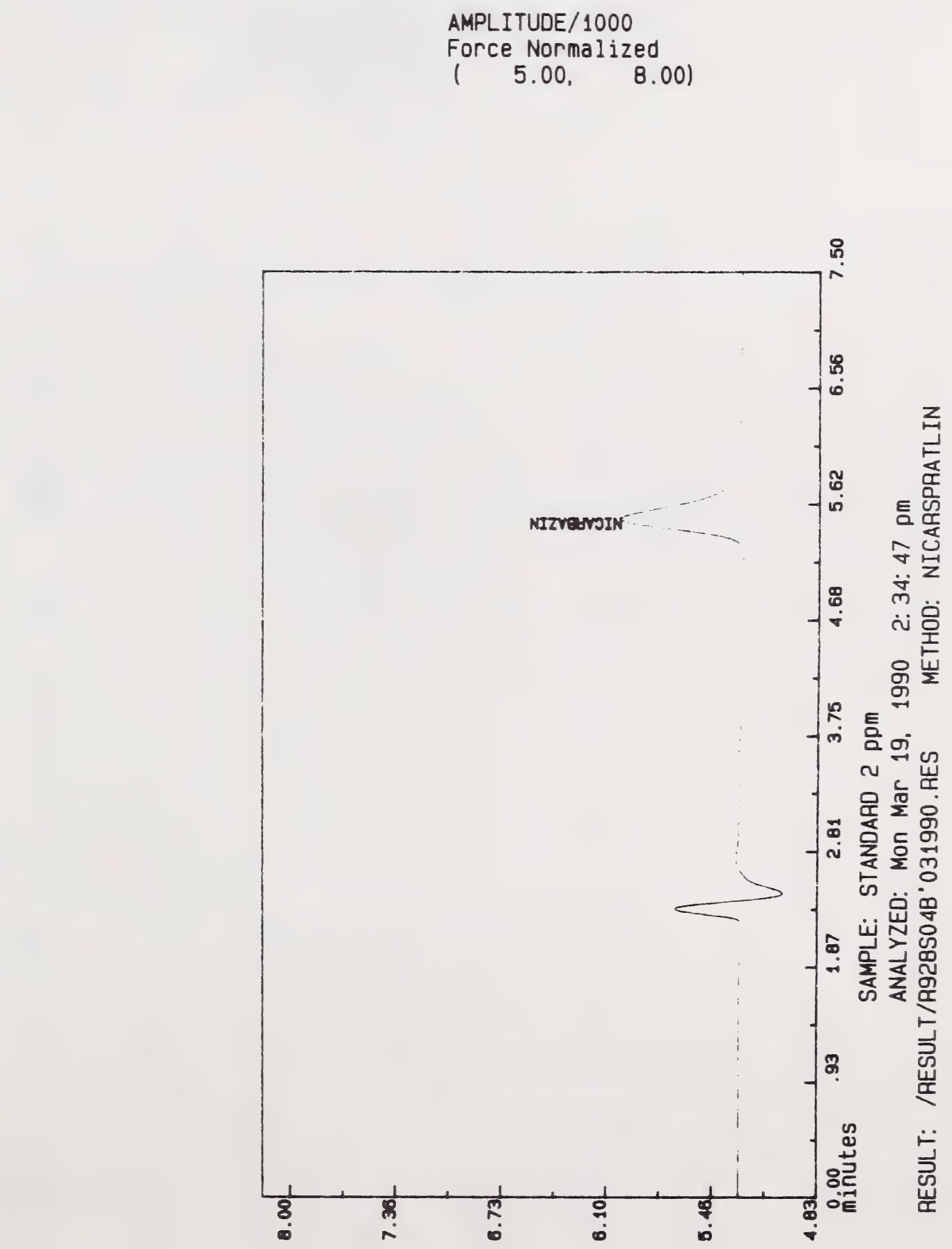
F. ANALYTICAL QUANTITATION (Continued)

4. Sample Chromatograms



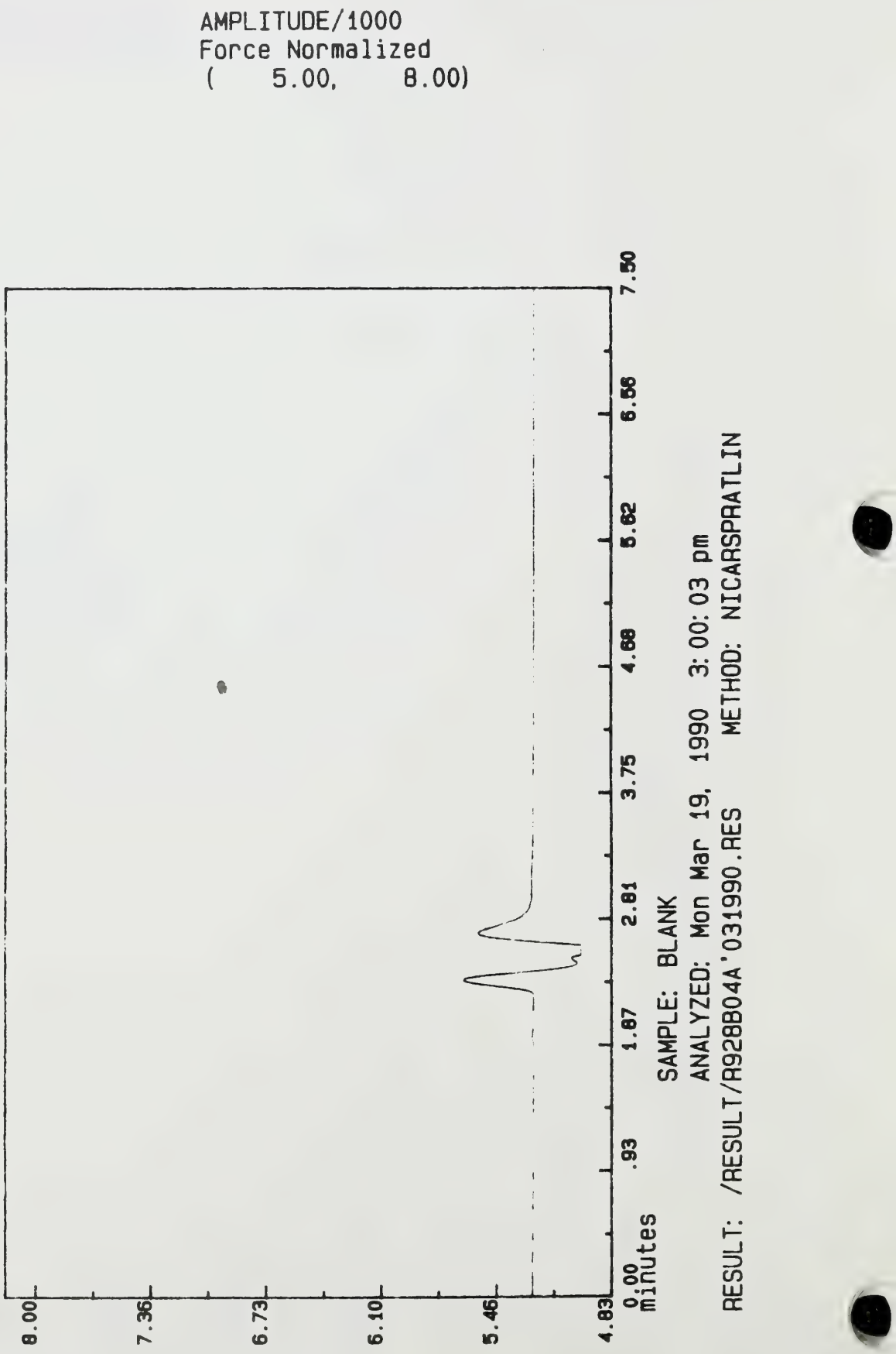
I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



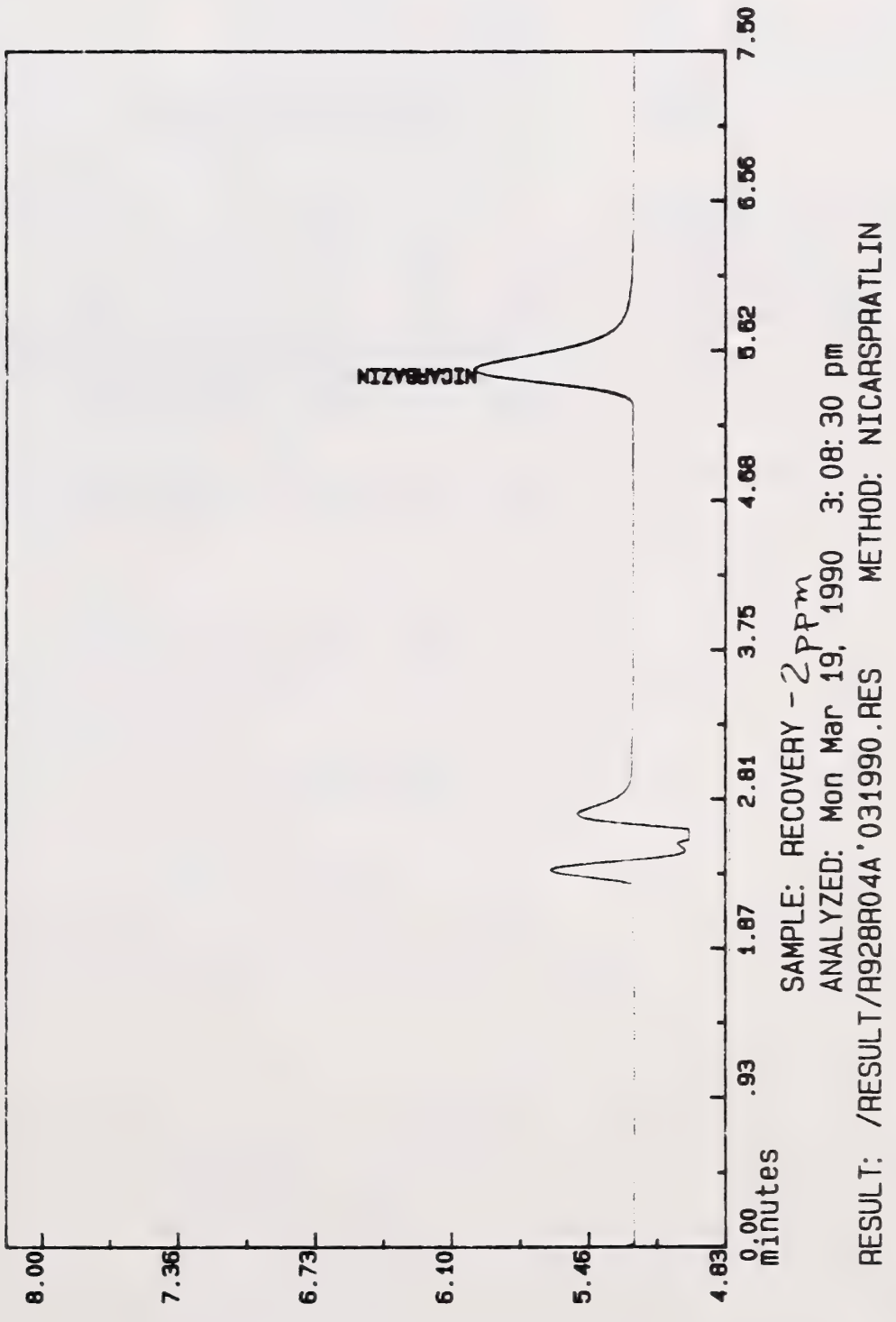
I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. By an acceptable means, measure the peak area of the 0, 2, 4, and 6 μg/mL HPLC standards (section D.2.c). Using the peak area and associated μg/mL values, construct a standard calibration curve by least squares computation, as indicated in the Chemistry Quality Assurance Handbook, Volume I, 1.5.67.

b. According to the following equation, calculate percent recovery (R_i) in the 2 μg/mL fortified control tissue.

$$R_i = \frac{C_r \times V \times AF \times 100}{\mu g \text{ fortified}}$$

where:

C_r = concentration of nicarbazin recovery sample as determined from the standard curve μg/mL.

V = final volume in mL (normally 10 mL).

AF = aliquot factor (normally 1.0).

c. Determination of the concentration (ppm) of nicarbazin tissue samples is accomplished by comparison with the standard peak areas and adjustment for the mean percent recovery of the fortified untreated control samples, if less than 100%, as shown below.

$$\text{ppm nicarbazin} = \frac{C_s \times V \times AF \times Fdnc \times 100}{W \times R_a \times 0.7089}$$

where:

W = weight (g) of tissue extracted.

R_a = last 10 running average percent recovery.

C_s = concentration of nicarbazin sample as determined from standard curve (μg/mL).

Fdnc = actual fraction of DNC in the nicarbazin reference standard (0.674 to 0.730).

0.7089 = theoretical fraction of DNC in the nicarbazin reference standard.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Nicarbazin in Poultry Tissues		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Acetonitrile Hexane Ethyl acetate Methanol DMF	Flammable. Vapors are irritating and may be absorbed through the skin, producing toxic effects.	Work in an efficient fume hood. Use plastic gloves when adding solvent and shaking the funnels.
4. Disposal Procedures	Organic solvents	(see above)	Store in the nonchlorinated solvent waste container until disposed of by contractor or in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Nicarbazin Analysis*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET FOR NICARBAZIN ANALYSIS

Analyst:_____ Analyst Code: [ ]

Date: _____

CONDITION 15 cm x 4.6 mm ID Zorbax
ODS–C–18 Column
Mobile Phase: 25% H_2O in CH_3OH (V/V)
Column temperature: 40°C
Flow: 0.8 ml/min (usual pressure 1000 psi, 500 – 2500 psi acceptable)
Injection volume 10 μl

NOTE: Evaporation Step – DO NOT ALLOW SAMPLE TO GO TO DRYNESS

SAMPLE IDENTIFICATION:	CRITICAL CONTROL POINTS

PROCEDURE KEY:	Sample Weight Grams	Spiking Volume ml	Shaking Time in Min	Final Volume ml	Peak Hght or Area	PPM found or % Recovery	Comments
STANDARD 0 PPM							
STANDARD 2 PPM							
STANDARD 4 PPM							
STANDARD 6 PPM							
BLANK	10.0 gms		3 min	10 ml			
RECOVERY 2 PPM		2 ml					
1 Sample #							
2 Sample #							
3 Sample #							
4 Sample #							
5 Sample #							
6 Sample #							
7 Sample #							
8 Sample #							
9 Sample #							
10 Sample #							
11 Sample #							
12 Sample #							
13 Sample #							
14 Sample #							
15 Sample #	V		V	V			

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Peformance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatbility %CV*	*Reproducibility %CV*
Nicarbazin†	0-6.0 ppm	80-110	10	15‡

† DNC component.
‡ Reproducibility is estimated value; actual value to be determined after validation study.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	10.0 ± 0.1 g
b. Volume of DMF	2.0 ± 0.1 mL
c. Volume of residual solution	10.0 ± 0.2 mL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Duplicate sets of standard curves on each of three days at 0, 2.0, 4.0, and 6.0 ppm.

ii. Phase II: Duplicate self-fortified recovery samples using chicken liver tissue spiked at 0, 2.0, 4.0, and 6.0 on three days.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation. Twelve chicken liver tissue samples, ranging from 0.4 to 6 ppm, including blanks, submitted by supervisor or Laboratory QA Officer.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst.

ii. Random replicates or blind samples may be chosen by the supervisor or Laboratory QA Officer.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) CUSUM charts.

(c) All recovery values.

(d) Running average, standard deviation, and CV for all recoveries.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Liver.

b. Sample receipt size: Sufficient for all quantitative analyses and sample reserved for confirmation (at least 500 g).

c. Condition upon receipt: Chilled or frozen.

d. Sample storage:

i. Time: Not determined; stability study required.

ii. Condition: Not determined; stability study required.

6. Sample Set

a. Blank.

b. Fortification at 2 ppm.

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 0.1 ppm.

b. Lowest reliable quantitation (LRQ): 0.4 ppm.

II. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory

Extracts from the determinative method are separated from interferences by reverse phase gradient HPLC. A diode array detector is used to determine the UV spectra of the suspected nicarbazin peak, which are compared to those of a standard.

2. Applicability

This method is applicable to all tissues referenced in the determinative method, section A.2, NIC-1.

B. EQUIPMENT

1. Apparatus

Refer to determinative method, section B.1, NIC-2.

2. Instrumentation

a. Liquid chromatograph: Hewlett-Packard Model 1090M, equipped with autoinjector, binary gradient option, and diode array detector (DAD), controlled by a Hewlett-Packard Model 79994 HPLC Chemstation.

b. Analytical column: 25 cm × 4.6 mm id, containing 5 μm Econosphere C_{18} spherical particles (Alltech Associates).

NOTE: Equivalent instrumentation may be substituted for that specified if necessary.

C. REAGENTS AND SOLUTIONS

Refer to determinative method, section C, NIC-3.

D. STANDARDS

Refer to determinative method, section D, NIC-4.

II. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP

Refer to determinative method, section E, NIC-5 through NIC-8.

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. HPLC parameters.

i. Flow rate: 1.0 mL/min.

ii. Injection volume: 25 μL.

iii. HPLC gradient.

NOTE: Since the system varies depending upon the matrix, it is recommended that the gradient system be used only if interfering peaks were observed when running the analysis with the isocratic system. If the isocratic system is the choice, use 75:25 methanol/water as the eluent. Otherwise the following conditions could be used as guidelines for a gradient system.

(a) Initial mixture: 60:40 methanol/water.

(b) Initial hold: 7 minutes.

(c) Gradient 1: Ramp to 80:20 in 4 minutes.

(d) Hold 1: 5 minutes.

(e) Gradient 2: Ramp to 60:40 in 4 minutes.

(f) Hold 2: 3 minutes.

b. DAD parameters.

i. Signal A: 345 $\pm$ 2 nm (4 nm bandwith).

ii. Signal B: 223 $\pm$ 2 nm.

iii. Signal C: 258 $\pm$ 2 nm.

iv. Signal D: 313 $\pm$ 2 nm.

v. Signal E: 373 $\pm$ 2 nm.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

vi. Reference signal for signals A-E: 550 ± 50 nm.

vii. Spectrum range: 210-450 nm.

viii. Store spectrum: Peak controlled, threshhold 1 mAU.

ix. Stop time: 16 min (peak elutes at 14 min).

c. Instructions for data acquisition.

Set up instrument operating parameters as described above. Allow the DAD to warm up at least 30 min before attempting analysis. After sample run is complete, use Chemstation software to integrate chromatographic peaks generated by signals A-E and produce a report of the results. In addition, a background-corrected spectrum of the peak of interest should be generated and printed out to allow a visual comparison of spectra.

2. Required Samples

A confirmation set consists of an external standard, a recovery, a tissue blank, and the presumptive positive sample from the determinative method. The external standard and recovery should be prepared at concentrations similar (±50%) to those found in the sample.

3. Criteria for Confirmation

a. Retention time specificiation. Retention time of the sample peak should match that of the standard and recovery within a 1% tolerance.

b. Wavelength ratio specification. All ratios of signals B-E to A for the sample must match those of the standard or recovery within 10%.

Absorbance Ratios for Nicarbazin

*Wavelength**	*Absorbance (Normalized)*
345	1.00
225	0.58
260	0.070
315	0.53
375	0.48

*Specified wavelength ± 2 nm.

c. Spectral comparison specification (visual confirmation). The background-corrected spectrum of the sample peak must match those of the standard or recovery with respect to location of absorbance minima and maxima. In addition, the overall shape of the sample spectrum must match that of the standard or recovery.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

4. Interferences

Some extracts from the determinative procedure may contain a number of compounds that can interefere in the absorbance range of 220-260 nm when isocratic (75:25 methanol:water) conditions are used for eluting. The gradient used has proven adequate for separating these interferences when using the specified column. Substitution of a different column may require development of a different gradient for adequate separation.

H. HAZARD ANALYSIS

Refer to Determinative Method, section H.

I. WORKSHEET

The worksheet on the facing page, *Nicarbazin HPLC-DAD Confirmation Data Form,* can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

NICARBAZIN HPLC-DAD CONFIRMATION DATA FORM

Sample Number: _____ **Tissue:** _____

Analyst: _____ **Date Run:** _____

Peak ht/area

Sample	Ret. Time	345	225	260	315	375
Blank						
Standard						
Recovery						

Pk ht/Area Ratio (relative to 345)

	225	260	315	375	Spectrum shape OK?
Blank					
Standard					
Recovery					

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 4 ppm.

b. No false negatives at 4 ppm.

2. Critical Control Points and Specifications

NOTE: Sample cleanup and preparation follow the same criteria described in the determinative method.

Record	*Acceptable Control*
DAD warmup	> 30 minutes
DAD zero	Prior to start of each injection (automatic with Chemstation software)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Inject standard (4 μg/mL) to verify HPLC, DAD, and integration software operating parameters.

ii. Phase II (Optional—at discretion of supervisor): Analyze two sets of validation samples on separate days. Each set consists of a standard at 4 μg/mL, a reagent blank, 1 blank poultry liver, and 3 poultry liver blanks fortified at 4 ppm.

iii. Phase III: Analyze a standard at 4 ppm, a blank poultry liver, a blank liver fortified at 4 ppm, and 3 unknowns fortified at approximately 4 ppm.

b. Acceptability criteria.

The analyst must demonstrate the ability to meet confirmation criteria specified in sections F.3 and F.4 for all samples analyzed.

4. Intralaboratory Check Samples

a. System, minimum contents:

i. Frequency: One sample quarterly per analyst.

ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer.

b. Acceptability criteria.

Ability to confirm samples using criteria specified in sections F.3 and F.4.

5. Sample Acceptability and Stability

a. Sample storage stability: Not determined.

b. Condition upon receipt: Cold.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	Refer to section F.2.
7. Sensitivity	a. Lowest detectable level (LDL): Not applicable. b. Lowest reliable confirmation (LRC): < 4 ppm.

NITROSAMINES/ONE TRAP GC-TEA MINERAL OIL VACUUM DISTILLATION ANALYSIS

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 5
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 10
G. Calculations 12
H. Hazard Analysis 13
I. [Reserved]
J. Quality Assurance Plan 14

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This procedure utilizes the Thermal Energy Analyzer, a device manufactured by Thermo Electron Corporation that provides detection of nitrosamine compounds. USDA Frying Protocol must be strictly followed before weighing sample for analysis.

A weighed sample of approximately 25 g is placed in a 500 mL flask and covered with mineral oil. The flask is connected, through a trap in liquid nitrogen, to a vacuum system. Under vacuum, the flask is heated slowly to 120° C and the volatile material is trapped. See Figure 1 on the following page.

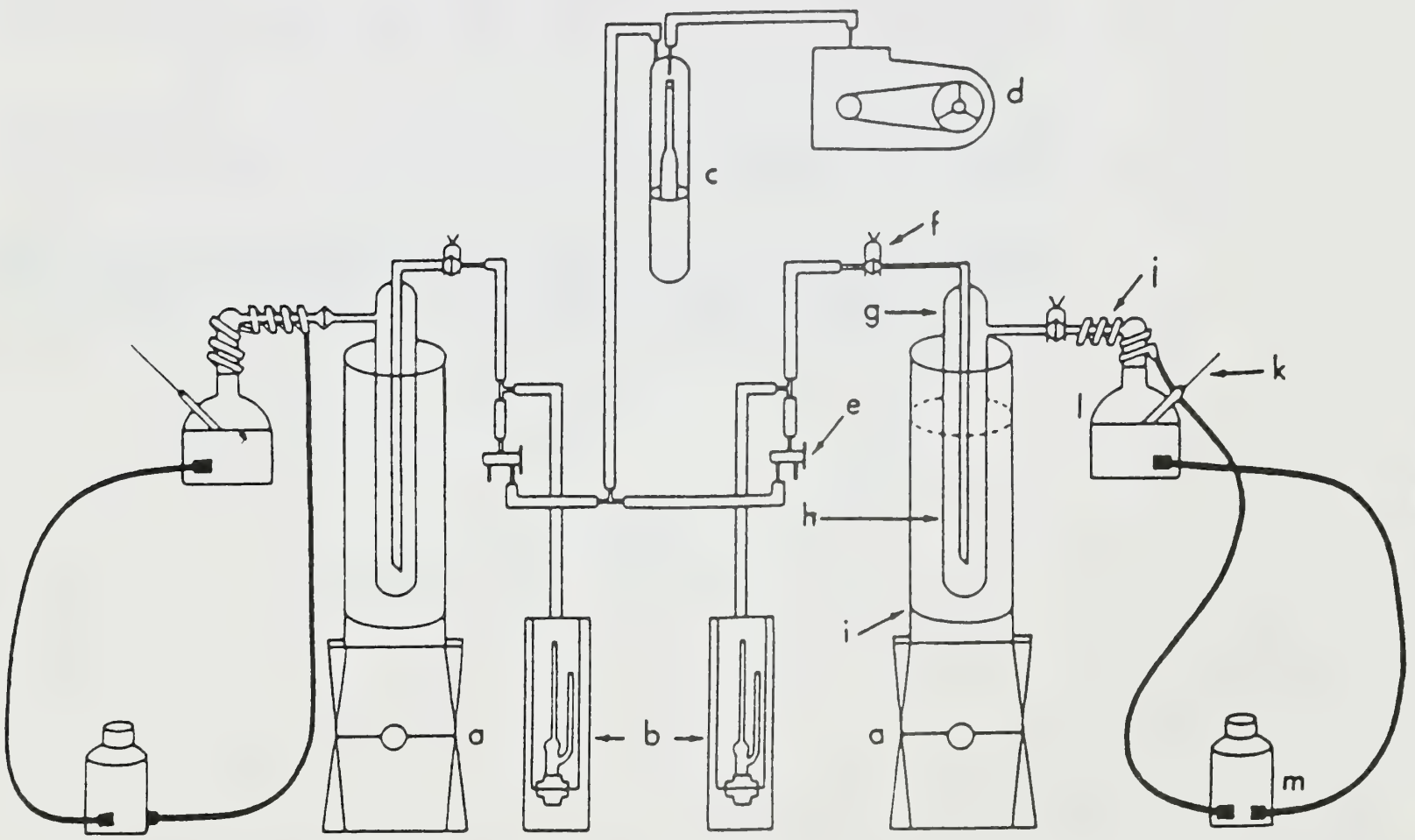
The trap and adapter are rinsed with methylene chloride. The washings and aqueous distillate are collected in a separatory funnel. The distillate is extracted several times. The combined extracts are dried with anhydrous sodium sulfate.

Under controlled conditions, the extract is concentrated to 1.0 mL. Suitable standard solutions are injected into the gas chromatograph attached to the TEA. The sample extract is injected and the readout compared to the readout from the standard solution injection.

DETERMINATIVE METHOD

B. EQUIPMENT

Figure 1



Pumping and Distillation Assembly
(Dual system. Single distillation setup may be used.)
a—laboratory jack; b—McLeod gage; c—vacuum controller; d—vacuum pump
e—3-way stopcock; f—pinch clamp; g—vapor trap; h—liquid nitrogen;
i—Dewar flask; j—heating tap; k—thermometer (−20° C to 150° C);
l—500 mL boiling flask with thermometer well; m—power stat.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

1. Apparatus

a. Boiling flask, 500 mL with thermometer well and 24/40 standard taper neck. Arthur H. Thomas Co. catalog number 4871-G-31 or equivalent.

b. Thermometer, −20° C to +150° C, 75 mm immersion. Fisher Scientific Co. catalog number 14-985-56 or equivalent.

c. Vapor traps, Arthur H. Thomas Co. catalog number 9466-R75, or equivalent. These traps are custom-fitted at both ends with 14/35 inner ground glass taper joints, Kontes 661250-1435, or equivalent.

d. Adapter (flask to trap), fitted with standard 24/40 joint to flask and 14/35 outer taper joint. Custom-made from a 24/40 adapater inlet, Kontes 180000-2440, and taper joint, Kontes 661500-1435, or equivalent.

e. Adapter (trap to vacuum pump), L-shaped, made from 14/35 outer taper joint, Kontes 661500-1435, or equivalent.

f. Vacuum pump, Welch Duo Seal, catalog number 1400B, 25 L/min free air displacement. Fisher Scientific Co. catalog number 1-096 or equivalent.

g. Stopcock, 3-way, with Teflon plug size 2. Kontes Glass Co. catalog number K-82300 or equivalent.

h. Vacuum tubing, 5/16″ bore. Fisher Scientific Co. catalog number 14-175D or equivalent.

i. Vacuum controller, cartesian type. Cole Parmer Instrument Co. catalog number 909 or equivalent.

j. McLeod gauge, shielded. Roger Gilmont Instruments catalog number G1400B or equivalent.

k. Heating mantle, 500 mL. Kontes Glass Co. catalog number K-726000 or equivalent.

l. Dewar flask, 4 L capacity, 17 cm od, 15 cm id, and 33 cm high. Fisher Scientific Co. catalog number 10-195D or equivalent.

m. Laboratory jack, Lab Lift. Fisher Scientific Co. catalog number 14-673-10 or equivalent.

n. Autotransformer, Powerstat type 3PN117B. Arthur H. Thomas Co. catalog number 9461-F-15 or equivalent.

o. Separatory funnel, 125 mL capacity. Kimble Products catalog number 29048-F or equivalent.

p. Funnel, Buchner, 60 mL capacity, coarse porosity fitted disc. Kontes Glass Co. catalog number K-955000 or equivalent.

q. Evaporative concentrator, Kuderna-Danish, 250 mL capacity, 24/40 standard taper column connection, 19/22 lower standard taper joint. Kontes Glass Co. catalog number 570001 or equivalent.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

r. Concentrator tube, size 425, 19/22 standard taper joint, 4 mL capacity, Kontes Glass Co. catalog number K-570050 (or equivalent) with 19/22 standard taper stopper. Kontes Glass Co catalog number K-850500 or equivalent.

s. Distillation column, Snyder, with 24/40 standard taper joint, 3 sections, size 121. Kontes Glass Co. catalog number K-503000 or equivalent.

t. Water bath, Freas Bath. GCA-Precision Scientific catalog number 66566 or equivalent.

u. N-Evap concentration equipment with water bath. Supplied with water bath #A-11151, Teflon-covered needles #10603, and thermometer #1111. Organomation Associates or equivalent.

v. Miscellaneous laboratory items, including but not restricted to: Teflon sleeve for standard taper joints; pinch clamp No.18, Arthur H. Thomas Co. catalog number 2841-A-19 or equivalent; extension-type pinch clamp No. 18, Arthur H. Thomas Co. catalog number 2841-E-77 or equivalent; hypodermic syringes; Pasteur pipettes; etc.

2. Instrumentation

a. Gas chromatograph, Shimadzu GD-4C PR 6 with column oven autocooling, temperature programmer, removable analytical cartridge, and including: injection port, temperature controller for injection port, and automatic door opener. Glass-lined heated transfer line from gas chromatograph to TEA. Dual column system with differential flow controllers, rotameters, and pressure gauges. Glass columns 2.7 m × 3 mm id (5 mm od) or equivalent. Recorder, R 11 m, single pen, 1 mv full scale. The gas chromatograph and its accessories are available from Shimadzu Scientific Instruments. Equivalent gas liquid chromatographic equipment can be substituted for the Shimadzu equipment.

b. Thermal Energy Analyzer (TEA) Model 502 with one direct sample injection mode and two gas chromatograph interface modes. This is the only sole source item in this list and is essential to this particular analysis. The TEA was obtained from Thermo Electron Corportation.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Paraffin-based mineral oil, white, heavy, Saybolt Viscosity 335/350 laboratory grade. Fisher Scientific catalog number 0-120 or equivalent.

b. Liquid nitrogen.

c. Methylene chloride. Burdick & Jackson, pesticide quality, or equivalent.

d. Anhydrous sodium sulfate analytical reagent grade. Mallinckrodt catalog number 8024 or equivalent, available from Arthur H. Thomas Co.

e. Carborundum, #12, granules. Arthur H. Thomas Co., catalog number 1590-33 or equivalent.

f. Mercury, reagent grade. Fisher Scientific Co. catalog number M-141 or equivalent.

g. Iso-octane, purified grade.

h. Sodium hydroxide, pellets, certified ACS. Fisher Scientific Co. catalog number S-318 or equivalent.

i. Column-packing material for GLC: 10% carbowax 20 M and 5% KOH on Analab AB 100/120 support or equivalent.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

a. Stock standard solution of N-dipropylnitrosamine, 5 μg/mL, in iso-octane. Obtained directly after clearance through FSIS, by writing to:

Mr. Steve Graves
NCI Chemical Carcinogen Repository
Midwest Research Institute
425 Volker Boulevard
Kansas City, Missouri 64110

b. Stock mixed standard solution of N-nitrosamine compounds, 5 μg/mL of each compound of interest in iso-octane, to be obtained directly from NCI after clearance through FSIS.

2. Preparation of Standards

a. Working level mixed standard solution. Dilute solution, section D.1.a, with methylene chloride (1:20).

b. Working solution of N-dipropylnitrosamine, used as internal standard. Dilute solution, section D.1 b, with methylene chloride (1:10).

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Distillation

CAUTION: The following procedures contain a number of hazardous steps. All basic safety precautions should always be used. A precautionary technique for preventing injury from imploding Dewar flasks is to wrap the outside of the flask with monofilament (glass fiber) tape. Extreme care should be exercised in handling nitrosamines or solutions of nitrosamines. These compounds are potent carcinogens. Wearing of rubber gloves is not, of itself, protection from nitrosamines. Every person handling nitrosamines, in any form, should read carefully references listed in sections F.3.h through F.3.j.

NOTES: N-nitrosamines are degraded by ultraviolet (UV) light and exposure of extracts or standard solutions to sources such as sunlight and fluorescent lighting should be avoided.

All reagents used in these analyses must be checked prior to use to assure that no extraneous compounds are present.

All glassware should be thoroughly cleaned, then rinsed with methylene chloride prior to use.

a. Place a 25 g sample in the boiling flask (refer to section B.1.a). Add 0.5 mL of solution of dipropylnitrosamine in methylene chloride (0.5 μg/mL); add 2.0 mL of a 0.2N solution of NaOH; cover the sample with 25.0 mL of mineral oil.

b. Wet inside of trap with about 2 mL of distilled water. Connect trap to vacuum line and raise Dewar flask so that trap is at least ¾ immersed in liquid nitrogen. Allow trap to come to equilibrium in the liquid nitrogen. Attach boiling flask and adapter to the trap. Position heating mantle on the flask. Apply vacuum and check for leaks as system comes to operating pressure. Maintain vacuum on sample 10 min before applying heat.

   NOTE: Vacuum leak difficulties are sometimes encountered when Teflon sleeves are used with standard taper joints. If necessary, vacuum grease can be used instead of Teflon sleeves.

c. Raise the temperature in the boiling flask, as indicated by thermometer in oil-filled well, from ambient to 120° C over a period of 55-60 min.

   NOTE: Two mineral oil distillation assemblies are connected to one vacuum pump, item B.1.e. See Figure 1. A higher vacuum (i.e., lower pressure than 2 torr) is maintained throughout sections E.1.c and E.1.d.

d. At the end of the heating period, lower the heating mantle away from the flask. Allow the flask 15 min to cool while the vacuum is maintained.

e. Carefully release the vacuum and lower the Dewar flask away from trap. Disconnect flask and invert adapter. Allow the trap to warm until the contents are liquid.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Transfer, Extraction, and Drying

NOTE: Subsequent sections describe washing or rinsing of trap, and extractions in separatory funnels. In the washing or rinsing of trap, a one-minute agitation of the contents is required to assure the best possible washing of all interior surfaces. All extractions in separatory funnels require one minute of vigorous shaking, followed by ample time for complete separation of the layers before removing the lower layer.

a. Rinse the adapter with 10 mL of methylene chloride collecting the rinisings in the trap.

b. Transfer distillate and methylene chloride washing to a 125 mL separatory funnel.

c. Rinse the trap with 15 mL of methylene chloride: 5 mL through the stem, 10 mL through the body of tube.

d. Transfer the methylene chloride to the separatory funnel and extract the aqueous distillate by shaking for 1 min.

e. Allow phases to separate. Transfer lower methylene chloride layer to a second 125 mL separatory funnel.

f. Perform sections E.2.c through E.2.e a total of three times.

g. Set up the Buchner funnel with 30 g of anhydrous sodium sulfate. Wash the anhydrous sodium sulfate with 25 mL of methylene chloride.

h. Set up a Kuderna-Danish flask fitted with concentrator tube. (Refer to section B.1.r).

i. Suspend the Buchner funnel containing the washed sodium sulfate over the Kuderna-Danish flask so that all liquid filtered through will be collected in the flask.

j. Dry pooled methylene chloride in second separatory funnel by passing it through washed sodium sulfate into Kuderna-Danish flask.

k. Rinse second separatory funnel with 25 mL of methylene chloride. Pass it through the sodium sulfate into Kuderna-Danish flask.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Extract Concentration

CAUTION: Several precautions are necessary in this section. Perhaps the most important one is: *From this point on, do not allow water to get into the extract.* Anytime the apparatus is subjected to condensation or is exposed to steam or other water vapor, caution is necessary to avoid water on ground joints or other entry into the extract.

a. Add one grain of carborundum to the Kuderna-Danish concentrator and fit the flask with a Snyder column.

b. Suspend the Kuderna-Danish apparatus in a 57°-63° C water bath. Adjust the temperature and/or depth of immersion of the apparatus, so that the volume of the extract will be reduced to 4.0 mL or less in 30 min to 1 hr. Water level must remain below the ground glass joint.

   NOTE: Water baths are available in many varieties. The one listed is a specific kind. It contains heated water under a relatively tight top. The top is fitted with concentric rings, so that a flask may simply be placed on a hole of appropriate size. Other baths may be substituted if adequate results are obtained.

c. Remove the apparatus from the bath, wipe dry, and allow to cool to ambient temperature.

d. Transfer the concentrator tube from the Kuderna-Danish apparatus to the N-Evap system with the water bath set at 35°-40° C. Further reduce the volume of the extract to 1.0 mL over a period of approximately 30 min.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Gas Chromatographic Separation

CAUTION: Gas chromatographic conditions are extremely important. Resolution between N-nitrosopiperidine and N-nitrosopyrrolidine must be at least 0.8. Conditions must be at least this good before using the chromatograph, must be checked periodically, and when column degradation or other conditions cause the GLC to fall below these specifications, a new column or returning must be provided. Operating conditions are shown in section F.2 below and an equation for calculating resolution is presented in section G.

a. Inject 6.0 microliters of the working mixed standard solution (0.25 μg/mL of each nitrosamine of interest). Obtain from this injection, the retention time and response of each compound of interest.

NOTE: As of this date, the list of nitrosamines of interest includes dimethylnitrosamine, diethylnitrosamine, dipropylnitrosamine, dibutylnitrosamine, N-nitrosopiperidine, N-nitrosopyrrolidine, and N-nitrosomorpholine. Dipropylnitrosamine is also used as an internal standard.

b. Inject 6.0 microliters of the concentrated sample extract. Obtain from this injection the retention time, and response of each compound of interest that is present.

c. Calculate the amount of each compound of interest present in the sample extract, and relate the amount to its level in the original sample, i.e., convert nanograms in the injection to parts per billion (ppb) in the original sample.

2. Gas Liquid Chromatographic (GLC) Parameters

Typical GLC conditions:

a. Injector: 185° C.

b. Column: 165° C.

c. Recorder: 1 mv.

d. Chart speed: 5 mm/min.

e. Carrier gas: N_2, 40 cc/min

f. Column: 2.7 m × 3.0 mm id packed with Analab AB 100/120 mesh coated 10% Carbowax 20 M and 5% KOH or equivalent.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. References

a. Havery, D. C., Fazio, T., and Howard, J. W. ''Survey of Cured Meat Products for Volatile N-Nitrosamines: Comparison of Two Analytical Methods.'' Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, 1978.

b. Gough, Terry A., Webb, Kenneth S., Pringer, Martin A., and Wood, Beryl J.A. ''Comparison of Various Mass Spectrometric and a Chemiluminescent Method for the Estimation of Volatile Nitrosamines,'' *J. Agric. Food Chem.*, Vol. 25, No. 3, 1977, 663-667.

c. Fine, D. H., Rounbehler, D. P., and Oettinger, P. C. ''A Rapid Method for the Determination of Sub-Part-per-Billion Amounts of N-Nitroso Compounds in Foodstuffs,'' *Analytical Chimica Acta*, 78(1975) 383-389.

d. Private communications to Mr. E. L. Greenfield from Dr. W. Fiddler, USDA, SEA, ERRD, Philadelphia, PA. Nov. 1978.

e. Private communications to Mr. E. L. Greenfield from T. Fazio, D. Havery, Dept. of HEW, FDA, Washington, DC, Dec. 1978.

f. National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens. Department of Health, Education, and Welfare Publication (NIH) 75-900 (1975).

g. Steere, N. V. ''Safety in the Chemical Laboratory CXVI—Occupational Safety and Health Standards Adopted for Fourteen Carcinogens.'' *J. Chem. Educ.* 51 (6), 1974, A322-A325.

h. Gough, T. A., Webb, K. S., and McPhail, M. E. ''Diffusion fo Nitrosamines through Protective Gloves.'' Presented at IARC Meeting, Durham, NH, August 1977; IARC Publication No. 19, 1978.

i. Walker, E. A., Castegnaro, M., Garren, L., and Pignatelli, B. ''Limitations to the Protective Effect of Rubber Gloves for Handling Nitrosamines.'' Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, 1978.

j. Sansone, E. B. and Tewari, Y. B. ''The Permeability of Laboratory Gloves to Selected Nitrosamines.'' Presented at IARC Meeting, Durham, NH, August 1977. IARC Publication No. 19, 1978.

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Chromatographic column should not be used when the resolution between N-nitrosopiperidine and N-nitrosopyrrolidine, calculated as follows:

$$R = \frac{T_2 - T_1}{\frac{1}{2}(W_1 + W_2)}$$

is less than 0.8.

When R = Resolution

T_1 = Retention time (mm) N-nitrosopiperidine

T_2 = Retention time (mm) N-nitrosopyrrolidine

W_1 = Peak width at base (mm) N-nitrosopiperidine

W_2 = Peak width at base (mm) N-nitrosopyrrolidine

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

One-Trap Mineral Oil-Vacuum Distillation—TEA Nitrosamine Procedure for Bacon (Prefried and Ground).

2. Required Protective Equipment

Safety glasses, protective gloves (impermeable to dichloromethane), lab coat, shield.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
(m) Working level mixed standard solution. Dilute—etc. with methylene chloride	The nitrosamines are potent carcinogens and the solvent itself is suspected of promoting tumors.	This dilution must be done in a fume hood being cautious not to contaminate the area.
D. Distillation		
NOTE: Two mineral oil distillation assemblies are connected to one vacuum pump.	Frostbite: Liquid nitrogen can readily boil over and spatter. Cuts: The distillation apparatus is fragile and is easily broken during assembly. Implosion: The Dewar flasks and distillation apparatus operating under vacuum can implode violently.	Check glassware for cracks or flaws. Use protective tape around vacuum glassware where feasible. All work should be performed behind a safety shield and the distillation assembly should not be repositioned once the system is under vacuum.

NOTE: This section should be re-read whenever an analyst is resuming this analysis.

4. Disposal Procedures

Nitrosamine solution	See above.	Store in a prominently labeled container until disposal by contractor.
Methylene chloride	See above.	Store with chlorinated waste until disposal by contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards§

Compound	*Analytical Range (ppb)*■	*Acceptable Recovery (%)*†‡	*Repeatability CV (%)*	*Reproducibility CV (%)*
DMNA	3-16	80-100	≤13	≤16
DENA	4-16	80-100	≤12	≤12
DBNA	4-17	80-100	≤14	≤14
NPIP	4-15	80-100	≤10	≤11
NPYR	3-18	80-100	≤11	≤11
NMOR	4-16	80-100	≤9	≤10

§Obtained from collaborative study.
■Range of collaborative study. Upper limit for method is greater than stated.
†Range for each nitrosamine recovery may be 70-110%.
‡CV on internal standard recoveries = ≤9 (for DPNA only); ≤8 for all others.

NOTE: The correlation coefficient of linear regression of fortified levels and determined levels should be 0.95 for DMNA, DENA, DBNA, NPIP, NPRY, NMOR, and DPNA.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample arrival.	
i. Temperature	25°-60° C.
ii. Regular bacon	18-24 slices to the lb
iii. Slab	Cut 10 slices to the inch (about 20 per lb)
iv. Labeled package received	Thin or thick slice, do not analyze.
b. Frying protocol.	
i. Weight of bacon used in frying	2 lb, 1¾ lb if proximate analysis is not run.
ii. Bacon slices	Randomly selected.
iii. Skillets	Calibrated daily, to produce a minimum temperature of 340°-345° F (171°-174° C) in the oil. Written records to be kept for each skillet. Readjustments and calibration until required temperature is obtained.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iv. Frying	Three minutes on each side for each individual strip. Begin timing when first strip is laid in skillet. At the end of exactly 3 minutes each strip is flipped over in the same sequence they were placed in the skillet.
v. Excess fat	Removed by blotting with paper towels.
vi. Fried sample	Ground after freezing, using a 11/32″ grinder plate, or Waring blender-type food processor, or equivalent.
vii. Initial setup only	170°-175° C.
viii. Maximum temperature of distillation	Ambient to 120° C in 1 hr.
ix. Vacuum on system maintained after heat is removed	Not less than 15 min.
x. Water bath	Concentrate to about 4 mL in ½ hr, not to exceed 1 hr maximum; water bath at 60° C ± 3° C.
xi. Final volume	1.0 mL (4 mL to 1 mL under stream of nitrogen 35°-40° C, not to exceed 1 hr).

NOTE: If internal recovery is not in the proper range, the sample is to be reanalyzed.

4. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Standard curves are not prepared in this analytical procedure.

ii. Phase II: Fortified samples—4 levels (7, 10, 15, and 20 ppb), 3 acceptable replicates each, over a minimum of 4 days, with blanks.

iii. Phase III: Check samples for analyst accreditation.

(a) 15 samples from FSIS Science Eastern Laboratory.

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Initially, minimum of 1 per set, reduced to 1 per week per analyst.

ii. Blind samples (requires ''dummy'' forms) or random replicates chosen by supervisor after initial analysis. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Control chart on difference between replicates.

(c) All percent recoveries.

(d) For all recoveries: the running average, standard deviation, and coefficient of variation.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Bacon (sliced, slab), other nitrite-cured products.

b. Sample receipt size: $\leq$2 lbs (1.75 lbs if proximate analyses are not required).

c. Condition upon receipt: Cold.

d. Sample storage:

i. Time: Up to 6 months (after frying).

ii. Condition: Frozen.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Set	a. External standard (mixed standard). b. Recovery sample fortified with mixed standard (or the nitrosamine of interest). c. Blank tissue fortified with the internal standard. d. Samples processed the same day.
7. Sensitivity	a. Lowest detectable level (LDL): Refer to 7.c below. b. Lowest reliable quantitation (LRQ): Refer to 7.c below. c. Minimum proficiency level (MPL). i. DENA, DBNA, NPIP, NMOR: 4 ppb. ii. DMNA, NPYR: 3 ppb.

NITROSAMINES/LOW-TEMPERATURE VACUUM DISTILLATION PROCEDURE

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 5
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 9
G. [Reserved]
H. Hazard Analysis 13
I. [Reserved]
J. Quality Assurance Plan 14

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

This procedure uses a low-temperature distillation technique developed by N.P. Sen (refer to section F.8) to recover the volatile N-nitrosamines from various meat products. Detection and quantitation are accomplished by the use of GC-TEA (refer to method NTR1). An additional column cleanup of the extract is adequate for mass spectral confirmation. USDA Frying Protocol must be strictly followed before weighing the sample for analysis.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Glassware catalog numbers refer to Kontes catalog TG-50 unless otherwise noted. Equivalent glassware may be substituted.

a. Distillation apparatus—refer to Figure 1 on the facing page.

i. All glass, flash evaporator, vertical, with adjustable heating bath (Buchler PTFE-1 GN, or equivalent.)

ii. 2 L round-bottom flask with 24/40 joint (K-601001).

iii. 250 mL round flask with 24/40 joint (K-601001).

iv. Needle value to adjust air bleed [Pyrex rotoflo (Corning 7740), or equivalent].

v. Ice bath with circulating water pump (Little Giant 501003, or equivalent).

vi. Vacuum pump, minimum 25 L/min capacity (GCA/Precision Scientific, Vac Torr D-25, or equivalent).

vii. Vacuum control cartesian (Gilmont C-2100, or equivalent).

viii. Vacuum gauge capable of monitoring pressure of 20 Torr. A manometer (Dwyer Series 1222 model M-1000, or equivalent) provides satisfactory service. Requires calibration with barometer of MacLeod gauge immediately before use.

b. Extraction and cleanup apparatus.

i. Syringe, all glass, 50 mL capacity.

ii. 250 mL graduated mixing cylinder (K-482000).

iii. 500 mL separatory funnel with Teflon stopcock (K-636030).

iv. Glass filtering funnel, 150 mL with 6 mm coarse porosity fritted disc (K-955000).

c. Evaporative concentrators.

i. 250 mL Kuderna Danish evaporative concentrator (K-570001).

ii. 500 mL Kuderna Danish evaporative concentrator (K-570001).

iii. 4 mL graduated concentrator tube (K-570050-425).

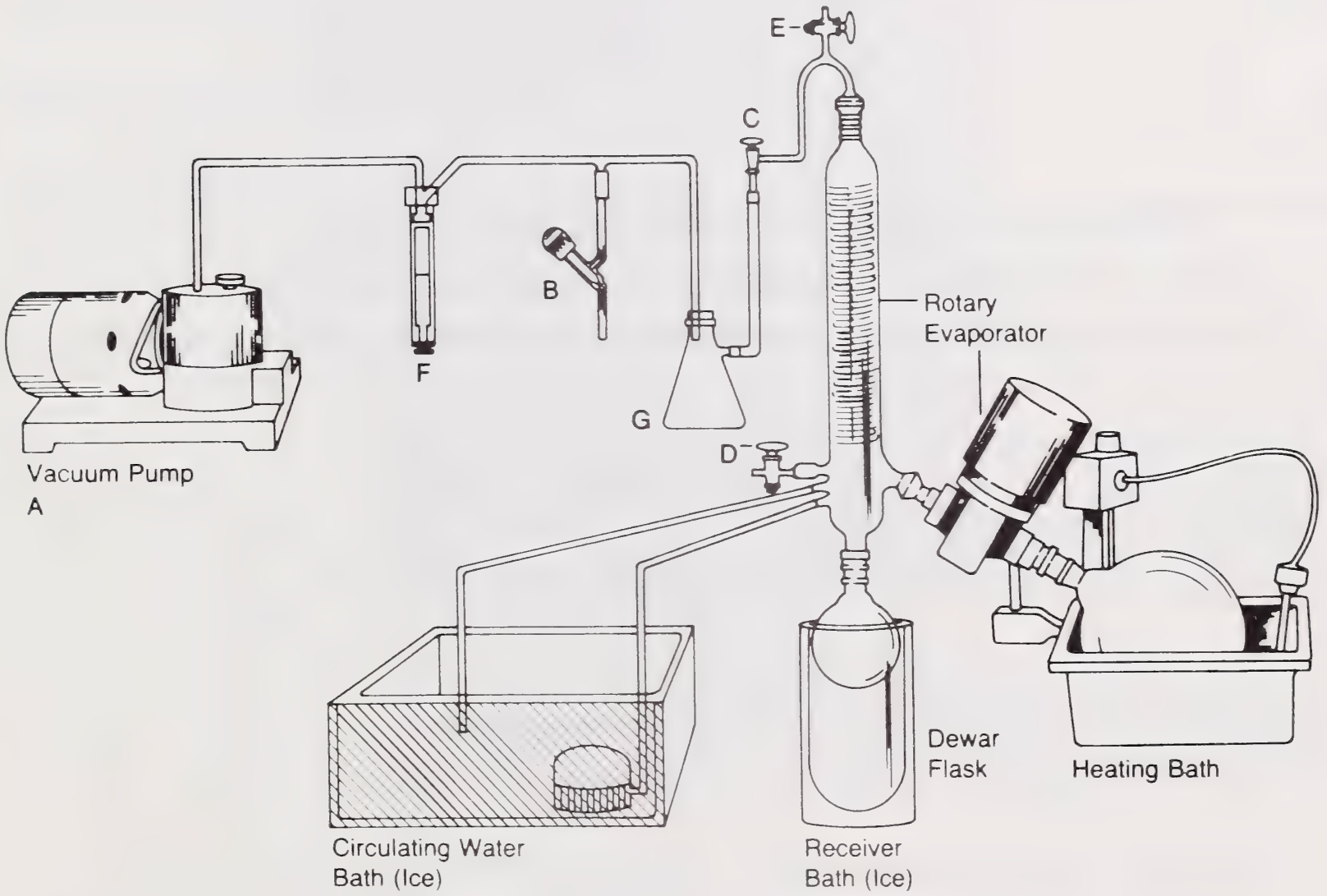
iv. Snyder column, large 3-section with 24/40 joint (K-503000-0121).

v. Snyder column, micro 3 section with 14/20 joint (K-569251).

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

Figure 1—Distillation Apparatus



A—Vacuum Pump
B—Variable Air Bleed
C—Isolation Valve
D—Vacuum Take-Off (Calibration)
E—Vent
F—Vacuum Regulator (Cartesian)
G—Trap

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

	d. Chromatographic apparatus. i. Chromatographic column, 10.5 mm id × 250 mm length with 200 mL reservoir (K-420280-213). ii Chromatographic column 22 mm id × 300 mm length (K-420540-233). iii. Pasteur pipet, disposable, 9 inch (K-88350). e. Water bath, circulating, with temperature controller, (Precision model 260, or equivalent).
2. Instrumentation	a. GC/TEA Shimadzu 4 CM gas chromatograph, or equivalent, interfaced to Thermo Electron 502 thermal energy analyzer. b. GC/MS Pye Unicam 104 interfaced to AEI MS30 by all-glass jet separator. c. GC/MS—Hewlett Packard 5992 with capillary interface and splitless injector.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solutions List

a. Dichloromethane (DCM). Concentrate 200 mL of each lot to 0.5 mL and check for interfering peaks. Extract with equal volume 6N HCl prior to use. Discard the HCl layer.

b. Pentane. Concentrate 100 mL of each lot to 1 mL and check for interfering peaks.

c. Methanol, anhydrous.

d. Hexane.

e. Acetone.

f. Water, glass-distilled. Do not pass through ion exchange resins.

g. Potassium hydroxide, reagent grade. Prepare 3N and 1N solutions.

h. Hydrochloric acid, concentrated, reagent grade. Prepare 6N solution.

i. Phosphoric acid, reagent grade. Prepare 6N solution.

j. Alumina, basic activity I (Fisher Scientific Co.). Heat overnight at 300° C. Deactivate 6% (w/w) with distilled water. Store in stoppered container in desiccator. Alumina is suitable for chromatography if cleanup procedure (refer to section F.4), using 1.0 mL mixed nitrosamine std (~.25 μg/mL in DCM), yields at least 85% recovery of NDMA, NPIP, NPYR, and NMOR with no traces of these compounds in the first eluate, and reagent blank shows no interfering peaks.

k. Celite 545, not acid-washed (Fisher Scientific Co.). Activate overnight at 700° C. Store in stoppered container in desiccator. Celite is suitable for chromatography if cleanup procedure (refer to section F.5), using 1.0 mL mixed nitrosamine standard (~.25 μg/mL in DCM), yields at least 85% recovery for NDMA, NPYR, and NMOR, and reagent blank shows no interfering peaks.

l. Silicon carbide granules, 12 mesh (Hengar Granules), or equivalent.

m. Glass wool, fine, for column chromatography.

n. Ice cubes, crushed ice, or dry ice for cooling bath.

o. Sodium sulfate, anhydrous, granular, reagent grade.

NOTE: All solvents listed are glass-distilled (Burdick and Jackson Laboratories, Muskegon, MI).

DETERMINATIVE METHOD

D. STANDARDS

1. Source	N-nitrosamine standards to be obtained directly from IITRI, after clearance through FSIS, from: Program Manager, Chemical Respository, Illinois Institute of Technology Research Institute, 10 W 35th Street, Chicago, Illinois 60616.
2. Preparation of Standards	The stock mixed nitrosamine standard consists of an iso-octane solution containing approximately 5μg/mL of N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), N-nitroso-dibutylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR), and N-nitrosomorpholine (NMOR). A separate NPDA standard is provided for use as a surrogate. Prepare working standards for the determinative method at a concentration of about 0.25 μg/mL by diluting the stock standards. GC injection standards are prepared by diluting the stock mixed standard with dichloromethane. Fortification standards are prepared by diluting the stock mixed and NDPA standards with methanol. Standards for GC/MS confirmation are prepared by dilution of the stock standards with hexane to a concentration of 1-2 μg/mL or more.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Adjusting Distillation Procedures

a. Refer to Figure 1, section B. Assemble distillation apparatus as shown. Prior to running samples, adjust the vacuum regulator to maintain the desired pressure. Using a vacuum gauge or manometer, obtain a zero reading by attaching the gauge directly to the vacuum pump (A) and recording the pressure. Re-attach the vacuum pump to the distillation apparatus and attach the vacuum gauge to the vacuum take-off point (D). Turn on the pump and adjust the air bleed (B) until a gauge pressure reading 19-21 torr higher than the zero value is achieved. Adjust the vacuum regulator to maintain this pressure using the instructions provided with the instrument.

b. Fill water bath to a normal operating level and adjust the temperature regulator to maintain a bath temperature in the range of 45°-50° C.

2. Distillation

a. Weigh 25 g sample into a 2 L round-bottom flask. Add 200 mL 3N KOH and 1.0 mL NDPA (0.25 μg/mL in methanol).

b. Attach distilling flask to evaporator and 250 mL round-bottom receiver to condenser.

c. Immerse receiver in an ice bath (0°-3° C) and begin circulation of ice water through condenser.

d. Immerse distilling flask in heating bath at 45°-46° C, apply vacuum, and turn on rotary evaporator. [CRITICAL STEP: Temperature must be maintained at no lower than 45° C.)

e. Adjust air bleed (B), if necessary, to ensure that vapors condense no further than one-third the way up the condenser. If excessive foaming occurs, reduce vacuum with air bleed, as necessary, to prevent mechanical transfer of distillate, until foaming condition diminishes.

f. As distillation proceeds, increase vacuum until full vacuum (20 torr) is reached. Full vacuum should be reached within 10 min of onset of distillation.

g. Continue distillation until drip rate from condenser averages less than one drop per minute. Total distillation time should be about one hour.

h. When distillation is complete, turn off rotary evaporator. Disconnect vacuum, using isolation valve (C), release vacuum, using vent (E), and remove receiver.

i. Transfer contents of receiver to a 250 mL mixing cylinder.

j. Reattach receiver to condenser, and using a large syringe, thoroughly wash down the walls and coils of the condenser with about 160 mL DCM, taking care to wash out all remaining aqueous distillate. Remove receiver and save for following step.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Extraction and Cleanup

a. Acidify aqueous distillate with 4 mL concentrated HCl and mix by shaking. Note volume, then transfer to a 500 mL separatory funnel.

b. Fill mixing cylinder with DCM rinse from condenser. Add additional DCM, if necessary, until volume is equal to that of acidified distillate.

c. Mix by shaking and add to aqueous distillate in separatory funnel. Extract distillate by shaking vigorously for 1 min.

d. Allow to stand until layers separate, then drain DCM layer into a second 500 mL separatory funnel.

e. Extract distillate with a second equal volume of DCM as before, and pool extracts. Discard distillate.

f. Wash combined extracts with 50 mL 6N HCl, shaking vigorously for 1 min. After phases separate, drain lower layer into a third 500 mL separatory funnel.

g. Backwash acid twice with 50 mL portions DCM, shaking 1 min each time.

h. Add first backwash to extract in third funnel. Save second backwash to extract KOH solution in step k.

i. Wash DCM in third funnel with 50 mL 1N KOH, shaking 1 min.

j. Allow phases to separate; then drain DCM phase through a 60 mm glass fritted funnel containing 30 g sodium sulfate (prewashed with 30 mL DCM) into a 500 mL Kuderna Danish concentrator fitted with a 4 mL graduated concentrator tube. This is necessary to remove traces of water from the extract.

k. Drain second acid backwash into third funnel now containing 50 mL KOH. Shake 1 min and allow phases to separate. Drain DCM layer through sodium sulfate into concentrator.

l. Add 1 mL hexane and a silicon carbide granule, attach Snyder Column, and evaporate on a circulating water bath at 60°-65° C, to 4-5 mL at a rate not to exceed 3 mL per min. Allow vapor to condense completely. Remove concentrator tube, attach micro Snyder Column, and add a second silicon carbide granule. Immerse tip of tube in 60°-65° C bath and evaporate to about 0.8-0.9 mL (Critical Step—Do Not Evaporate with Nitrogen). Remove from bath and wash down column with a few drops DCM. Cool, and adjust volume to 1.0 mL with DCM. Stopper tube.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. GC/TEA Shimadzu 4 CM gas chromatograph, or equivalent, interfaced to Thermo Electron 502 thermal energy analyzer.

GC conditions: 2.7 m × 3 mm id glass column packed with 10% Cabowax 20 M + 5% KOH on 100/120 chromosorb PAW; injection port 200° C; interface 280° C isothermal; nitrogen carrier gas 25-30 mL/min TEA conditions: furnance 470° C; liquid nitrogen trap; initial and working vacuum settings and oxygen flow must be determined for particular instrument being used.

Recorder response should be > 10% for 1.5 ng N-nitrospyrrolidine. Resolution (R) between N-nitrosopiperdine and N-nitrosopyrrolidine should not be < 0.8 when calculated as follows:

$$R = \frac{T_2 - T_1}{\frac{1}{2}(W_1 + W_2)}$$

where T_1 and T_2 = retention times (mm) of N-nitrosopiperdine and N-nitrosopyrrolidine; W_1 and W_2 = peak width as base (mm) of N-nitrosopiperdine and N-nitrosopyrrolidine.

b. GC/MS Pye Unicam 104 interfaced to AEI MS 30 by all-glass jet separator.

GC conditions: 9 ft × 3 mm id glass column packed with 5% FFAP on 60/80 chromosorb W HP: injection port 160° C, column temperature 145° C isothermal; jet separator 180° C; helium flow 18 mL/min. MS parameters:

MS 30: Temperature at ion source = 200° C
Resolution of 10% valley definition = 3300
Temperature at jet separator = 180° C

c. GC/MS Hewlett-Packard 5890 gas chromatograph with split/splitless capillary inlet, interfaced via open-split interface to a Hewlett-Packard model 5970 Mass Selective Detector.

GC conditions: Column = HP-1 (bonded methyl silicone) capillary, 0.2 mm × 12 m × 0.33 μm thick. Carrier gas: helium; inlet pressure: 12 psi. Injection mode: Grob splitless; purge delay: 40 sec. Temperatures: inlet 150° C, interface 245° C. Column temperature program: Hold 2 min at 50° C; ramp to 110° C at 5° C/min; ramp to 245° C at 20° C/min; hold 1 min. Elution times: NPYR 6.9, NMOR 7.1, NDPA 7.7, NPIP 8.2, NDBA 13.3.

2. General Operations

Inject a suitable aliquot on GC-TEA and quantitate all nitrosamines. Use standards at ~.25 μg/mL in DCM for quantitation. NDPA recovery must be at least 80%, or distillation should be repeated. If samples require dilution for quantitation, dilute with n-pentane, *not DCM,* or additional cleanup for mass spectral confirmation will be compromised. If samples must be stored overnight prior to further cleanup, add sufficient n-pentane to bring volume to 4-5 mL.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

Samples containing any nitrosamine in concentrations equal to or exceeding 10 ppb are violative, provided that the presence of the nitrosamine can be positively confirmed by mass spectrometry. When a sample requires confirmation, the tissue blank and recovery that have been analyzed concurrently must also be analyzed as confirmation samples.

3. Mass Spectral Confirmation—Additional Cleanup

The nature and amount of interfering substances remaining in the concentrate after initial distillation and cleanup varies markedly from sample to sample. Most bacon samples will require further cleanup prior to successful confirmation by mass spectrometry. Cleanup procedures using alumina and acid-celite column chromatography have been investigated. Acid celite usually provides better overall cleanup for samples containing NPYR and NMOR, while alumina is more effective when NDEA, NDPA, NDBA, or NPIP are present. The column of choice for samples containing NDMA is best determined experimentally by the analyst.

4. Alumina Column Chromatography

a. Add a small glass wool plug to a 10.5 mm × 250 mm chromatographic column. Rinse column with 5 mL DMC, then 5 mL n-pentane, allowing rinses to drain. Close stopcock and add 15 mL pentane to column. Add 5 g alumina (use of a long stem funnel is helpful) and allow to settle. Wash down any alumina that may adhere to column walls with a small amount of n-pentane. Add a layer of sodium sulfate about 1 cm thick to top of column, then drain n-pentane to a level 1 mm above top of sodium sulfate. Add 3 mL n-pentane to column.

b. Using a Pasteur pipet, transfer sample (mixed with 3 mL pentane) to column. Rinse concentrator tube twice with 2 mL n-pentane, adding rinsings to column. If sample contains NDEA, NDPA, or NDBA, place a 250 mL Kuderna- Danish apparatus with 4 mL concentrator tube under column. Drain sample onto column at 60-80 drops/min until level is 1 mm above top of sodium sulfate. Wash down walls of column with 2-3 mL n-pentane and drain to same level.

c. Add 50 mL 20% DCM in n-pentane to column and elute at 60-80 drops/min until level reaches top of column. Momentarily stop flow and replace concentrator, if used, with another Kuderna-Danish apparatus. Add 60 mL DCM to column and drain into concentrator at 60-80 drops/min. If initial fraction was collected (for NDEA, NDPA, NDBA), add 25 mL DCM to that fraction. Add 1 mL hexane and a silicon carbide granule to all fractions collected and concentrate to 4-5 mL on a 55-60° C circulating water bath at a maximum rate of 3 mL/min. Concentrate to approximately 0.5-1.0 using a micro Snyder column as previously described.

5. Acid Celite Chromatography

a. Weigh 5.0 g celite into a 150 mL beaker. Add 7.5 mL 6N H_3PO_4 in approximately 2.5 mL portions, stirring vigorously with a glass rod to assure a uniform mixture that is fluffy in consistency.

b. Pack mixture into a 22 mm id × 300 mm chromatographic column containing a small glass wool plug. Compress celite to 4.5 cm, placing a small glass wool plug on top of absorbent.

c. Add 10 mL n-pentane to column, and drain until liquid layer is 1 mm above top of celite layer.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

d. Using a Pasteur pipet, quantitatively transfer sample onto column using sufficient pentane so that the volume of sample plus washes is approximately 10-12 mL.

e. Drain sample onto column at 60-80 drops/min, stopping when liquid level is 1 mm above celite.

f. Wash column walls with 3-4 mL pentane, draining to previous level.

g. Add 70 mL 2% DCM in pentane to column and drain at 60-80 drops/min.

h. Pass 60 mL DCM through column, collecting eluate in a 250 mL Kuderna-Danish apparatus.

i. Add 1 mL hexane and concentrate as described under alumina column chromatography.

6. Preparing Samples for Mass Spectrometry

Transfer concentrate from sections F.3 or F.4 to a 1 mL reaction vial or centrifuge tube graduated in increments of at least 0.1 mL. Adjust the volume of the sample so that its nitrosamine concentration matches that of the standard to be used for the confirmation. (For example, if the standard injected has a nitrosamine concentration of 5 μg/mL, a sample quantitated at 10 ppb will have to be reduced in volume to 50 μl). Concentrate the sample slowly, using a gentle stream of dry nitrogen.

7. Mass Spectrometry

Successful confirmation by mass spectrometry requires that both the GC retention and the monitored ion fragments of the sample match those of an injected standard. The possibilities of false positives should be compensated for by injections of a tissue blank and a recovery, respectively, in the same run. Both a magnetic sector instrument operating at medium resolution and a quadrupole have been used for confirmation. Parameters for both are listed below.

a. Confirmation parameters—AEI MS 30.

i. Calibration of the mass spectrometer.

The mass spectrometer should be calibrated with perfluorokerosene (PFK) or other suitable calibration compounds for the mass range of interest with the 10% valley definition resolution better than 3000. to separate 29.9980 to 30.0343 and 28.0188 to 28.0061 (H_2^+. ionized from the residual air in vacuum system).

ii. Confirmation of nitrosamines in study sample.

The gas chromatographic retention time of a purported nitrosamine in a study sample should be within $\pm$2% of the nitrosamine in the standard sample or fortified blank.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTIATION (Continued)

The presence of the molecular ion (M^+) (or quasimolecular ion $(M + H)^+$ in case of using chemical ionization mode) and the most significant characteristic fragment ions for nitrosamines at m/z 29.9980 (NO^+) and at least two other fragment ions must be detected with the signal to noise ratio larger than two. These additional two fragment ions are usually of the following ions: m/z 28.0188 (CH_2N^+), 29.0266 (CH_3N^+), 30.0343 (CH_4N^+), 41.0266 ($C^2H_3N^+$), 42.0344 ($C_2H_4N^+$),43.0422 ($C_2H_5N^+$), 44.0500 ($C_2H_6N^+$), and the $(M-NO)^+$, $(M-HNO)^+$, and $(M-H_2\ NO)^+$ ions.

b. Confirmation parameters—HP 5890/5970 MSD.

i. Calibration of mass spectrometer.

Successful autotune calibration must be achieved prior to attempting confirmation. Operate at the highest practical resolution.

ii. The CC retention time in the sample should be within 3% of the standard or recovery. A minimum of 4 characteristic ions, including the molecular ion, must be detected. Ions to be monitored for each compound are: NDBA (158, 116, 141, 84, 57, 30); NPIP (114, 55, 56, 42, 39, 30); NPYR (100, 68, 69, 42, 39, 30); and NMOR (116, 86, 56, 55, 41, 30). Confirmation requires that the ion intensity ratios relative to the molecular ion for at least 3 ions in the sample match those of the standard or recovery within ± 20%.

Table 1. Nitrosamine Confirmation Parameters‡

Compound	*RT*	*Monitored Ions (Relative Ratios)*					
NPYR	6.9	100(1.0)	69(0.1)	68(0.2)	42(0.9)	39(0.3)	30(0.5)
NMOR	7.1	116(1.0)	86(0.9)	56(2.8)	55(0.3)	41(0.5)	30(1.3)
NPIP	8.2	114(1.0)	56(0.5)	55(0.9)	42(2.0)	39(0.4)	30(0.6)
NDBA	13.3	158(0.1)	84(1.0)	116(0.2)	141(0.1)	57(0.9)	30(0.5)

‡Actual retention times and relative ion ratios will vary depending upon the condition of the column used and tune of the mass spectrometer.

8. References

a. Sen, N. P., Private Communication, Canadian Dept. of Health and Welfare, Food Research Division, Ottawa, Ontario.

b. Sen, N. P., et al., *J. Agric. Food Chem.*, Vol. 27, No. 6, 1979, pg. 1354-1357.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination of Nitrosamines by the Low-Temperature Vacuum Distillation Procedure.

2. Required Protective Equipment

Safety glasses, protective gloves (impermeable to dichloromethane), lab coat, shield.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
(a) N-nitrosamine standards etc. Dilute with methylene chloride	The nitrosamines are potent carcinogens and the solvent itself is suspected of promoting tumors.	This dilution must be done in a fume hood, being cautious not to contaminate the area.
D. Standards		
(b) Distillation	*Explosion:* The Dewar flasks and distillation apparatus operation under vacuum can implode violently. *Cuts:* The distillation apparatus is fragile and is easily broken during assembly.	Check glassware for cracks or flaws. Use protective tape around vacuum glassware where feasible. All work should be performed behind a safety shield and the distillation assembly should not be repositioned once the system is under vacuum.

4. Disposal Procedures

Nitrosamine solution	See above.	Store in a prominently labelled container until disposal by contractor.
Methylene chloride	See above	Store with chlorinated waste until disposal by contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Compound	*Analtyical Range (ppb)†*	*Acceptable Recovery (%)‡*	*Repeatability CV (%)*
DMNA, NPYR, NMOR	10-20	70-110	8
DBNA	10-20	55-95	15

†For confirmation purposes.

‡With average not less than 80% based on last 10 recoveries. Not less than 80% for DPNA (up through TEA measurement—does not include GC/MS portion of methodology.)

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample arrival.	
i. Temperature.	25-60° C.
ii. Regular bacon.	18-24 slices to the lb.
iii. Slab.	Cut 10 slices to the inch (about 20 per lb).
iv. Labeled package received.	Thin or thick sliced, do not analyze.
b. Frying protocol.	
i. Weight of bacon used in frying.	2 lb, 1¾ lb if proximate analysis is not run.
ii. Bacon slices.	Randomly selected.
iii. Skillets.	Calibrated daily, to produce a minimum temperature of 340°-345° F (171°-174° C) in the oil. Written records to be kept for each skillet. Readjustments and calibration until required temperature is obtained.
iv. Frying.	3 min on each side for each individual strip. Begin timing when first strip is laid in skillet. At the end of exactly 3 min, each strip is flipped over in the same sequence they were placed in the skillet.
v. Excess fat.	Removed by blotting with paper towels.
vi. Fried sample.	Ground after freezing using an 11/32-inch grinder plate, or Waring blender-type food processor, or equivalent.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	Record	*Acceptable Control*
	vii. Temperature.	45-46° C.
	viii. Concentration method.	Do not use any inert gas (N_2) or air to assist in the evaporation. Use Kuderna-Danish evaporators, topped with micro Snyder columns, on a steam bath or water bath.

NOTE: If internal standard recovery is not in the proper range, the sample is to be reanalyzed.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—For this analytical procedure, there is no standard curve preparation. Pure standards are not taken through the method. This phase of familiarization is therefore waived.

ii. Phase II: Fortified samples—Blanks and 4 nominal levels at 7, 10, 15, and 20 ppb over a minimum of 4 days for DMNA, NPYP, and NMOR (20 samples).

iii. Phase III: Check samples for analyst accreditation.

(a) 15 samples from FSIS Science Eastern Laboratory.

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence offical analysis.

b. Acceptability Criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst.

ii. Blind samples (Requires ''dummy'' forms) or random replicates chosen by supervisor after initial analysis.

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer.

(a) All replicate findings.

(b) Control chart on difference between replicates.

(c) All % recoveries.

(d) For all recoveries: the running average, standard deviation, and coefficient of variation.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	b. Acceptability criteria. If unacceptable values are obtained, then: i. Stop all official analyses by that analyst. ii. Investigate and identify probable cause. iii. Take corrective action. iv. Repeat Phase III of section J.3 if cause was analyst-related.
5. Sample Acceptability and Stability	a. Matrices: Bacon (slice, slab), other nitrite-cured product. b. Sample receipt size: ≤ 2 lbs (1.75 lbs if proximate analyses are not required). c. Condition upon receipt: Cold. d. Sample storage: i. Time: Up to 6 months (after frying). ii. Condition: Frozen.
6. Sample Set	a. External standard (mixed standard). b. Recovery sample fortified with mixed standard (or the nitrosamine of interest). c. Blank tissue fortified with the internal standard. d. Samples processed the same day.
7. Sensitivity	a. Lowest detectable level (LDL): 5 ppb. b. Lowest reliable quantitation (LRQ): 10 ppb. c. Minimum proficiency level (MPL): 10 ppb.

ORGANOPHOSPHATES

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 6
F. Analytical Quantitation 8
G. Calculations 11
H. Hazard Analysis 12
I. [Reserved]
J. Quality Assurance Plan 13

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Fifty grams of liver or muscle tissue are weighed into a centrifuge bottle. The weighed sample is fortified with ethyl parathion as an internal standard and blended. A recovery sample, fortified with the working standard solution, is analyzed in parallel. Tissue is blended, centrifuged, and filtered. The filtrate is extracted with an ethyl acetate/hexane mixture by blending and then centrifugation. The extract is concentrated, reconstituted to specific volume and partioned with acetonitrile. Further cleanup is accomplished by a charcoal column. The eluant is concentrated for GC analysis using a flame photometric detector.

The following organophosphates are quantitatively analyzed by this procedure: dioxathion, diazinon, methyl parathion, fenitrothion, malathion, ethyl parathion, ruelene, gardona, ethion, trithion, and coumaphos.

Other organophosphate pesticides such as chlorpyrifos, disulfoton, and trichlorfon can be qualitatively and quantitatively recovered by this procedure, but GLC retention times may be overlapping. The organophosphate pesticides listed above are all resolved when instrumental parameters and methodology specifications described herein are followed.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Centrifuge bottles, 250 mL, polypropylene, screw cap, Scientific Products #C4162-250, or equivalent.

b. Tissuemizer, Tekmar Model STD, or equivalent.

c. Centrifuge, Sorvall RC 5, or equivalent.

d. Round bottom flask, 500 mL, Fisher Scientific #10-067G, or equivalent.

e. Filter paper, Whatman, 2 V fluted, 18-24 cm, or equivalent.

f. Rotary evaporator, Buchler Flash, evaporator, Buchler Instruments, or equivalent.

g. Volumetric flask, Class A 100 mL, 10 mL, 50 mL; Scientific Products #F4665-100, #F4665-10A, #F4665-50, or equivalents.

h. Screw-cap scintillation vials, Curtin Matheson Scientific #250-332, or equivalent.

i. Graduated concentrating tubes, 4 mL, 10 mL, Kontes #570050-0425, #570050-1025, 15 mL, Curtin Matheson #053-132 or equivalents.

j. Pipet, 1 mL, 10 mL, Class A, Fisher Scientific #13-651A, #13-651K, or equivalents.

k. N-Evap, Organomation Model 111 or 112, or equivalent.

l. Vortex mixer, Fisher Scientific #12-814, 12-810, or equivalent.

m. Disposable Pasteur pipets, 9″, Curtin Matheson #063172, or equivalent.

n. Silanized glass wool, Applied Science #14502, or equivalent.

o. Micropipet, 0.1 mL, Fisher Scientific #21-164-2H, or equivalent.

p. Syringes, 5mL, Curtin Matheson Scientific, glass with Luer-Lok Tip #221-465, needle (25 guage) #221-986, and 10 μl Hamilton #801, or equivalent.

2. Instrumentation

a. Gas chromatograph, Tracor Model #222, or equivalent, equipped with phosphorous/sulfur dual-flame photometric detector (sulfur detector is optional), nitrogen-phosphorous flame ionization detector.

b. Column: 6 ft × 4 mm id column, 10% OV-101 on Chromosorb W—High performance (80/100 mesh). Condition column at 275-280° C overnight @ 50 mL/min nitrogen flow.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

c. Alternate or confirmation columns.

i. 4% SE-30/6% OV-210 coated on Gas Chromosorb Q or Chromosorb W-High Performance 80/100 mesh.

ii. 5% or 10% OV-210 coated on Gas Chromosorb Q or Chromosorb W-High Performance 100/120 mesh.

iii 1.5% OV-17/1.95% OV-210 coated on Gas Chromosorb Q or Chromosorb W-High Performance 100/120 mesh.

NOTE: Tables of elution patterns for these three columns can be found in *Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Environmental Samples*—U.S. Environmental Protection Agency.

iv. 2% stabilized DEGS on Chromosorb W-acid washed 80/100 mesh. This column separates parent compounds from the oxygen-analogs. Also affects elution order of many of the organophosphates. *Pesticide Analytical Manual—Volume 1, Table 334-A*—Food and Drug Administration.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Ethyl acetate (EtAOc), pesticide quality (Burdick and Jackson, or equivalent).

b. Petroleum ether, pesticide quality (Burdick and Jackson, or equivalent).

c. Hexane, pesticide quality (Burdick and Jackson, or equivalent).

d. Acetonitrile, pesticide quality (Burdick and Jackson, or equivalent).

e. Acetonitrile saturated with petroleum ether.

f. Methylene chloride (CH_2Cl_2) (Burdick and Jackson, or equivalent).

g. Celite 545: Johns-Manville, Denver, CO 80217. Must be free of substances that interfere with GLC determinations. Remove electron capturing substances by slurrying with 1 + 1 hydrochloric acid-H_2O while heating on steambath. Wash with H_2O until neutral. Wash with methanol, then EtOAc, and then with hexane, and dry. Remove substances that interfere with phosphorus-selective GLC by heating at 600° C in a muffle furnace for at least 4 hr.

h. Charcoal: Nuchar SN, available from Eastman Kodak or Scientific Products. To cleanup charcoal sufficiently for use in charcoal column cleanup, slurry 200 g with 500 mL conc. HCl, cover with watch glass, and stir magnetically while boiling 1 hr. Add 500 mL H_2O, stir, and boil additional 30 min. Collect charcoal in Buchner funnel and wash with H_2O until washings are neutral to universal indicator paper. Dry at 130° C in a forced-draft oven overnight.

i. Magnesium oxide: Sea Sorb 43 (Westvaco #2641) (Fisher Scientific Co., catalog #D-120). Treat as follows: Slurry about 500 g with distilled H_2O, heat on steambath about 30 min, and filter with suction. Dry overnight at 105°-130° C and pulverize to pass #60 seive. Store in closed jar.

j. Charcoal absorbent mixture: Mix 1 part acid-treated charcoal, 2 parts hydrated magnesium oxide, and 4 parts Celite 545, acid-washed. Keep in a closed container. For example: 100 grams charcoal, 200 g hydrated MgO, 400 grams Celite 545.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Primary standards can be obtained from:

U. S. Environmental Protection Agency
Director, Environmental Monitoring Support Laboratory
Office of Research and Development
Cincinnati, OH 45268

2. Preparation of Standards

a. Standard stock solutions:

Weigh 40-50 mg of standard into a screw-cap scintillation vial (or other closable container). Dissolve with a small volume of ethyl acetate. Transfer to a 50 mL volumetric flask and dilute to volume. Store stock solutions in freezer.

b. Working standard solution (used as a fortification solution for recovery sample):

Determine the number of milliliters of each stock solution needed to approximate the working standard concentrations in Table 1 (refer to section F.2). Combine the appropriate amounts of each standard in a 100 mL volumetric flask and dilute to volume with ethyl acetate. Keep refrigerated when not in use.

c. Gas chromatography standard (for injection):

Dilute 0.5 mL of working standard solution to 10 mL with ethyl acetate.

3. Storage Conditions

Keep refrigerated when not in use.

4. Shelf Life Stability

Prepare fresh solution about every two weeks.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 50 g of liver or muscle into 250 mL plastic centrifuge bottle.

b. Spike samples with 1 mL of internal standard ethyl parathion. Spike recovery with 1 mL of recovery spiking solution (ethyl parathion alone, at working standard concentration).

c. Add 100 mL of mixture 70% EtOAc and 30% hexane, v/v, to each sample.

d. Blend sample for 1 min (tissuemizer).

e. Centrifuge for 10 min at 2,000 rpm.

f. Filter extract into a 500 mL round bottom flask. (2 V fluted filter paper 18 cm or 24 cm.) If blood has separated during centrifugation, do not let blood get into filter.

g. Add 100 mL of 70% EtOAc-30% hexane mixture to tissue and again tissuemize for 1 min.

h. Centrifuge at 2,000 rpm for 10 min.

i. Filter extracts into same 500 mL round bottom in step 1.f. (Combine the two extracts.) If blood has separated during centrifugation, do not let blood get into filter.

j. Evaporate extract to 90-95 mL on roto-evaporator (50°-60° C).

k. Transfer and dilute to volume with 70% EtOAc-30% hexane mixture in 100 mL volumetric flask while rinsing round bottom flask into volumetric. Stopper and mix well.

l. Transfer extract to plastic bottles with screw caps or 250mL centrifuge bottles.

m. Pipet 10 mL extract into graduated concentrator tube (15 cc). Place plastic bottles in freezer.

n. Reduce volume to approximately ½ mL on N-Evap.

o. Bring volume to 3 mL with petroleum ether. Mix well. (Use an additional 1-2 mL of petroleum ether for samples with excessive fat.)

p. Add 7 mL of acetonitrile (saturated with petroleum ether). Stopper and mix gently with a back and forth motion. Leave standing a few minutes to allow the layers to separate.

q. Aspirate and discard top layer (petroleum ether layer). This should eliminate some of the fat.

r. Add 2 mL of petroleum ether and repeat shakeout. Let separate. Aspirate and discard top layer.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

s. Concentrate on N-Evap. When volume reaches 2-3 mL, rinse sides with EtOAc. Continue to concentrate until volume is less than 1 mL.

t. Make volume to 1 mL with EtOAc. Stopper tubes and mix well on vortex mixer.

u. Mix charcoal absorbent mixture well before each use. Weigh 0.3-0.4 g of charcoal adsorbent mixture (refer to section C, Reagent and Solution List, item j) for each sample. Place a small plug of silane treated glass wool in a 9″ disposable Pasteur pipet. Add the adsorbent mixture to the pipet (column). Tap gently to settle. Add a small plug of glass wool on top of the absorbent bed to prevent disruption while adding solvent to the column.

v. Add 20 mL of eluting solvent (CH_2Cl_2) to a flask and place next to the sample flask. (Label flasks.)

w. Pre-rinse columns with 3 mL CH_2Cl_2 from the flask containing 20 mL $MeCl_2$. Let drain and discard CH_2Cl_2 eluate.

x. Collect extract from column in an Erlenmeyer flask. Place flasks under each tube to collect eluant. (Label flasks!)

y. Add 0.1 mL of the sample to column via disposable micropipet. Rinse pipet with 1 mL eluting solvent onto the column using a syringe.

z. Let drain to near top of column packing. Add eluting solvent to column. *Do not let columns go dry.* Add eluting solvent until the remainder of the 20 mL have been used. Approximately 30-45 min required.

aa. Concentrate on N-Evap. Transfer extract to concentrator tube. When volume reaches 1-2 mL, rinse empty flask with 2-3 mL of ethyl acetate and add to evaporating tube. Stopper second mix. Continue evaporation.

bb. Concentrate to 0.1 mL (do not evaporate to dryness). For FPD, make volume to 0.2 mL with EtOAc. Mix samples on vortex mixer. Stopper each tube. For FID, add 3-4 mL hexane, reconcentrate to 0.1 mL (do not evaporate to dryness). Make volume to 0.2 mL with hexane.

NOTE: A larger dilution may be made for use with automatic injectors.

cc. Inject appropriate volume of sample solution into the gas chromatograph using the conditions described in section E. q.

NOTE: Before injection of samples, it is advisable to ''condition'' the column packing by injecting a high concentration standard followed by 3-4 injections of the gas chromatography standard. This procedure reduces chances of adsorption of compounds at the low levels (especially polar compounds).

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. Phosphorus/sulfur, dual flame photometric detector (sulfur detector is optional):

Nitrogen carrier gas	80 mL/min
Hydrogen	150 mL/min
Air	60 mL/min
Oxygen	20 mL/min

NOTE: Set gas flow rates according to manufacturer's instructions if GC is other than model specified above.

Temp program	160° C, hold 2 min 160° C-250° C @ 8° C/min 250° C, hold 6-10 min
Injection temp	220° C
Flame photometric detector temp	190° C (should be 150°-160° C before flame is lit)

b. Nitrogen-phosphorous flame ionization detector:

Helllium carrier gas	40 mL/min
Hydrogen	3 mL/min
Air	80 mL/min
Temp program	160° C, hold for 1 min 160° C-250° C @ 8° C/min 250° C, hold 17-18 min
Injection temp	225° C
Nitrogen-phosphorus FID temp	300° C

NOTE: The air and/or O_2 to H_2 ratio can be critical for some organophosphate compounds. The air flow should be adjsted to produce the best response without increasing baseline noise.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

2. Column Treatment

Carbowax 20 M column treatment (refer to Figure 1 on following page):

The conditioned column is treated with Carbowax 20 M in the following manner: About 2″ (5 cm) of support material coated with 10% Carbowax 20 M is placed in the injection port area of the column and separated from the support in the packed column with a small amount of silanized glass wool. After installing the column in the instrument, raise the injection port temperature to 230° C and the column temperature to 225° C. These conditions are maintained overnight with a flow of 10-20 mL/min of nitrogen. After treatment, the column is removed from the instrument and the 2″ of carbowax material is removed and discarded. The column is reinstalled for analyses. The internal standard (ethyl parathion) should have a retention time of ca 9 min with a ca 40% full-scale deflection for a 4 μl injection at the concentration described in Table 1 below.

TABLE 1

Organophosphate	*Stock Solution Concentration*	*Working Std Concentration*	*GC Standard Concentration*	*Concentration in 4 μl Injection*
Dioxathion	1.0	54.00	2.70	10.80
Diazinon	0.8	15.98	0.799	3.20
Methyl parathion	1.0	19.30	0.965	3.86
Fenithrothion	0.8	16.23	0.812	3.24
Malathion	1.0	20.23	1.01	4.04
Ethyl parathion	0.8	15.94*	0.797	3.18
Ruelene	1.0	29.35	1.47	5.88
Gardona	1.0	51.11	2.56	10.24
Ethion	0.8	15.70	0.785	3.14
Trithion	0.8	24.11	1.21	4.84
Coumaphos	1.0	77.62	3.88	15.52

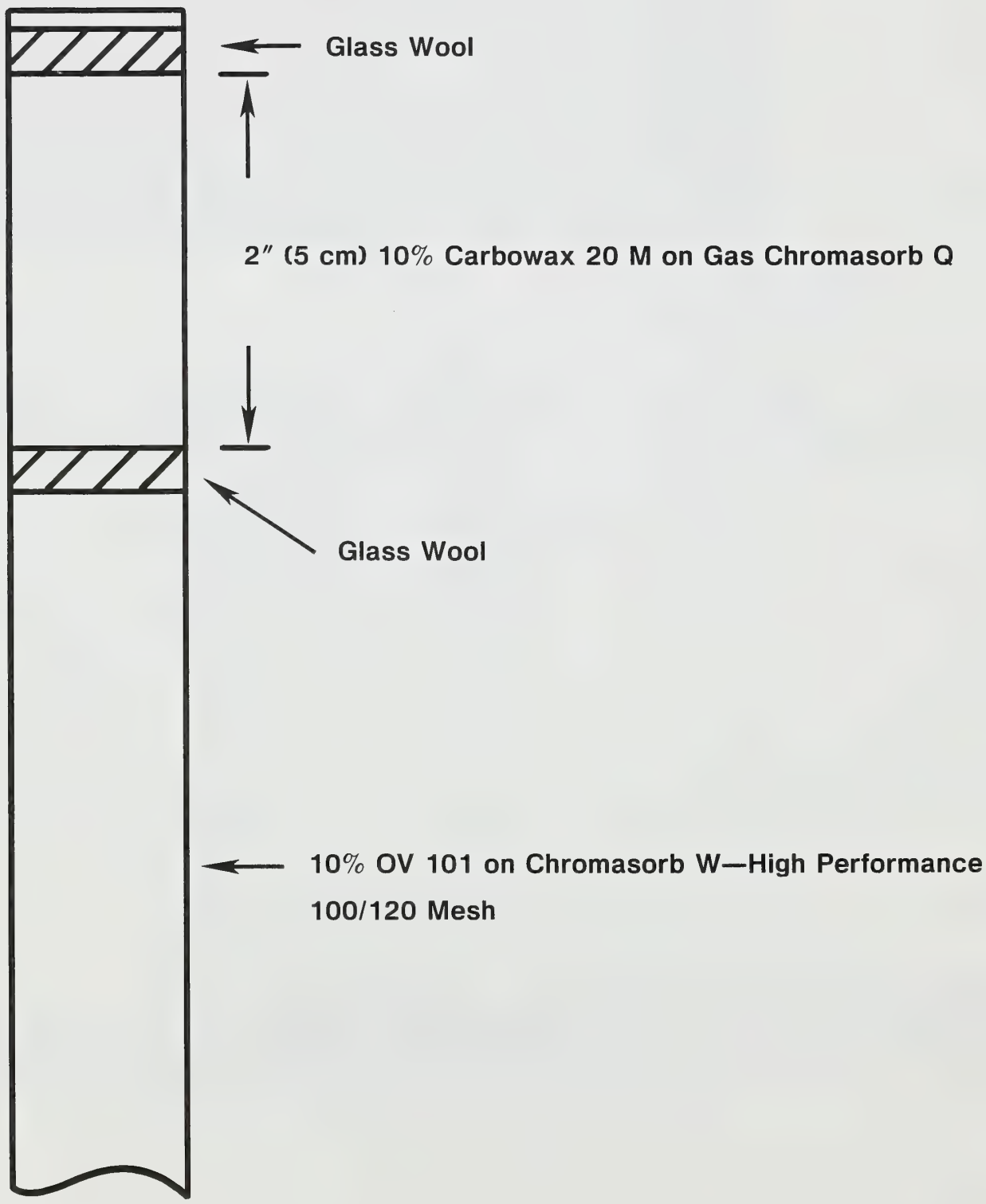
*Used as an internal standard

NOTE: The concentrations of the organophosphates are approximate. The analyst should adjust the concentrations, if needed, to get an optimum response and sensitivity for the instrument and detector used.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

Figure 1



Carbowax Conditioning of GC Column
for Organophosphate Residue Analysis

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

Concentration of an organophosphate (OP) present is calculated using either peak height or peak area as follows:

$$\text{(OP) ppm} = \frac{\mu\text{L std inj}}{\mu\text{L sample inj}} \times \frac{\text{pk ht (area) sample}}{\text{pk ht (area) std}} \times \frac{\text{Conc std } \mu\text{g/mL}}{2.5 \text{ g/mL}}$$

2. References

a. Storrher, R. W. ''A General Method for Organophosphorous Pesticide Residues in Non-Fatty Foods,'' *JAOAC* 54, 513-516(1971).

b. Watts, R. R., Storherr, R. W., and Pardue, J. R. ''Charcoal Column Cleanup Methods for Many Organophosphorous Pesticide Residues in Crop Extracts,'' *JAOAC* 52, 522-526 (1969).

c. Bowman, M. C., and Beroza, M. ''GLC Retention Times of Pesticides and Metabolites Containing Phosphorous and Sulfur on Four Thermally Stable Columns,'' *JAOAC* 53, 499-508 (1970).

d. Ives, N. Fred, and Guiffrida, Laura ''Gas-Liquid Chromatographic Column Preparation for Adsorptive Compounds,'' *JAOAC* 53, 973-977 (1970).

e. ''Manual of Analytical Methods for the Analysis of Pesticide Residues in Human and Enviromental Samples,'' U.S. Environmental Protection Agency Sections 4, B(1)-B(5).

f. ''Pesticide Analytical Manual—Volume 1'' Food and Drug Administration, Sections 230.00-2232.45, Table 334-A.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Organophosphate Pesticide Residue in Liver and Muscle Tissues.		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat, mask.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Ethyl acetate Petroleum ether Hexane Acetonitrile Methylene chloride	These solvents are all flammable and may produce toxic effects to the skin, eyes, and respiratory system.	Use these chemicals in an efficient fume hood away from electrical heating devices and open flames.
	D. Standards Spike samples with 1 mL of internal standard ethyl parathion	Organophosphate standards.	Fume hoods must be used since these compounds are highly toxic and are known to adversely affect the central nervous system. Some analysts feel even more comfortable using a glove box when handling these chemicals.
4. Disposal Procedures	Charcoal columns saturated with methylene chloride and extracted tissue	See above.	Allow columns to dry in the hood and dispose of charcoal packing by pouring into plastic bag and discarding.
	Reagent mixtures	See above.	Chlorinated and non-chlorinated mixtures should be segregated in different waste containers awaiting disposal by contractor or in-house specialist.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

Compound	*Analytical Range (ppb)†*	*Acceptable Recovery (%)‡*	*Repeatability %CV*	*Reproducibility %CV*
Dioxathion	> 0.10	60-110	< 12	< 20
Diazinon	> 0.03	60-100	< 12	< 20
Methyl parathion	> 0.04	70-110	< 12	< 20
Fenitrothion	> 0.03	80-110	< 12	< 20
Malathion	> 0.04	70-110	< 12	< 20
Ethyl parathion	> 0.03	70-100	< 12	< 20
Ruelene	> 0.06	70-100	< 12	< 20
Gardona	> 0.10	70-110	< 12	< 20
Ethion	> 0.03	60-110	< 12	< 20
Trithion	> 0.05	60-110	< 12	< 20
Coumaphos	> 0.15	60-100	< 12	< 20

†Chlorinated organophosphates may be analyzed in fat using method CHC3.

‡Other compounds can be detected and quantified but Performance Standards have not been established for them.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight.	50.0 g ± 0.1 g.
Internal standard.	Ethyl parathion, enough to produce ½ scale deflection in matrix.
Blend time.	1 min ± 10 sec.
Reducing volume.	Do not let go dry.
Final volume.	0.2 mL ± 0.02 mL

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

NOTE: Each set of samples or day's run must be represented by properly labeled chromotagram containing at a minimum the following items.

Date:

Analyst: Code:

Gas Chromatograph:

Detector:

Detector Temp:

Injection Temp:

Carrier Gas:

Column:

Flame Fuels:

Temperature Program:

Chart Speed:

- Each standard labeled with the following items:

  Standard identification

  Concentrations of compounds of interest
  Peak identifications
  Microliters injected
  Applicable measurements

- Each sample labeled with the following items:

  Identification
  Weight
  Dilutions
  Microliters injected
  Identification of chlorinated hydrocarbon peaks
  Applicable measurements
  Quantitative calculations

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—At least 3 levels, 3 replicates each, for each compound of interest.

(a) One-half minimum proficiency level.

(b) Minimum proficiency level.

(c) Twice minimum proficiency level.

ii. Phase II: Fortified samples must include at least 3 levels, with 3 replicates each, for each compound of interest over a minimum of 3 days.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples from FSIS Western Laboratory (samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

See section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Minimum of 1 per week per analyst, 20% of official samples analyzed (whichever is smaller).

ii. Blind samples—require ''dummy forms.''

iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer:

(a) All replicate findings.

(b) Running average difference between replicates.

(c) All percent recoveries.

(d) Running average, standard deviation, and CV for recoveries.

(e) Appropriate CUSUM charts.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver and muscle.

NOTE: Chlorinated organophosphates may be detected and quantified from fat using method CHC3.

b. Sample receipt size: Varied; enough to obtain matrix for all required screening, quantitation, and confirmation tests.

c. Condition upon receipt: Frozen.

d. Sample size:

i. Time: 14 working days.

ii. Condition: Frozen.

6. Sample Set

a. Blank tissue.

b. Recovery fortified at tolerance level (if applicable) or at MPL.

c. Sample(s) of same species.

7. Sensitivity

a. Lowest detectable level (LDL): Refer to 7.c below.

b. Lowest reliable quantitation (LRQ): Refer to 7.c below.

c. Minimum proficiency level (MPL):

i. Diazinon, fenitrothion, ethyl parathion, ethion: 0.15 ppb.

ii. Methyl parathion, malathion: 0.20 ppb.

iii. Trithion: 0.25 ppb.

iv. Ruelene: 0.30 ppb.

v. Dioxathion, gardona: 0.50 ppb.

vi. Coumpahos: 0.75 ppb.

ORGANOPHOSPHATE PESTICIDES IN FAT

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Sample Preparation 5
F. Analytical Confirmation Procedure 6
G. [Reserved]
H. Hazard Analysis 9
I. Worksheet 10
J. Quality Assurance Plan 13

CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory	This is a nonvalidated confirmatory method using a capillary column for separation and electron impact ionization for detection using the extract from the GPC, micro alumina column, or florisil column method. The absolute sensitivity of the confirmatory method varies from compound to compound. Selected ion monitoring is used to confirm the presence of all compounds listed in A.3 below.
2. Applicability	This method is applicable for the confirmation of organophosphate pesticides in animal fat.
3. Ions Monitored	The method is used to confirm the presence of the following compounds: ronnel, chlorpyrifos, chlorfenvinphos, stirophos, phosalone, coumaphos, linuron, endosulfan, and carbophenothion.

CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus	Refer to CHC3, section B.
2. Equipment	GC/MS: Hewlett-Packard 5970B MSD/5890 GC equipped with 30 m DB5 column (J & W Scientific, Inc.), 0.25 mm id, 0.25 micron film thickness, or equivalent.

CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Refer to CHC3, section C.

b. Dry nitrogen.

c. Hexane: Burdick & Jackson, distilled in glass, or equivalent.

d. Isooctane: Burdick & Jackson, distilled in glass, or equivalent.

CONFIRMATORY METHOD

D. STANDARDS

Preparation of Standards

a. Working standard will vary for each compound depending upon its sensitivity and confirmatory concentration.

b. Each standard will be diluted appropriately to match the concentration of the sample to be confirmed.

CONFIRMATORY METHOD

E. SAMPLE PREPARATION

Determination

a. Carefully evaporate to dryness the sample extract prepared for GC injection by the acetonitrile partition/Florisil column cleanup procedure or the micro alumina column procedure or the gel permeation chromatography method.

b. Dilute to 20 μL with isooctane. The volume may be varied such that injection of no more than 2.5 μL into the GC/MS produces a sufficient number of counts at each monitored ion so that confirmatory criteria can be met, using samples fortified at or below (about 80%) of the action level.

CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions.

i. Carrier gas: helium.

ii. Injection mode: Grob splitless.

iii. Purge flow: 40 mL/min.

iv. Purge off time: 1 min.

v. Injector temperature: 260° C.

vi. Column head pressure: 10 psi.

vii. Interface temperature: 285° C.

viii. Column temperature program:

(a) For compounds ronnel, chlorpyrifos, chlorfenvinphos, stirophos, phosalone, and coumaphos.

Temperature 1	40°, hold 1 min
Temperature rate 1	16° C/min
Temperature 2	200° C, no holding time
Temperature rate 2	6° C/min
Temperature 3	270° C, hold 7.33 min
Temperature rate 3	10° C/min
Final temperature	285° C, hold 1 min

(b) For compounds linuron, endosulfan, and carbophenothion.

Temperature 1	80° C, hold 2 min
Temperature rate 1	6° C/min
Temperature 2	250° C, no holding time
Temperature rate 2	16° C/min
Final temperature	285° C, hold 3 min

NOTE: It is necessary to experiment with different temperature programs to separate the analyte of interest from the background interferences. Additional sample clean-up is required if temperature programming alone cannot achieve the goal. The optimum GPC dump time should be determined to minimize the amount of late-eluting lipid getting into the sample.

CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

b. MS SIM acquisition parameters.

i. Start time: 14 min.

ii. Ion monitored: See F.3.e. below.

iii. Dwell time: 80 msec per ion.

iv. EM voltage: Autotune voltage + 200-400 V.

2. Instrument Performance Verification

Set up instrument as described in F.1 to monitor compounds of interest. Inject the standards and analyze results to verify that the system is functioning properly. If not, make any necessary adjustments in operating parameters or standard concentration; then reinject to verify performance.

3. Criteria for Confirmation

a. No false positives or negatives.

b. The specific ions listed in 3.e below must be present for the compound.

c. Retention time of the analyte peak must match that of the standard or the recovery within ± 5 seconds.

d. At least three ion ratios, relative to the most intense ion, must be within 20% of those found in the recovery sample spiked at the level detected in the suspect sample.

e. Ions monitored.

Compounds	*Ions Monitored/ Approximate Relative Ion Intensities*					
Ronnel	109 / 15	125 / 29	167 / 4.3	270 / 6.0	285 / 100	287 / 76
Chlorpyrifos	169 / 12	197 / 79	208 / 29	258 / 44	286 / 41	314 / 100
Chlorfenvinphos	170 / 12	204 / 8.0	267 / 100	295 / 31	323 / 92	325 / 64
Stirophos	109 / 68	157 / 3.7	204 / 5.0	240 / 6.7	329 / 86	331 / 100
Phosalone	121 / 53	154 / 25	182 / 100	184 / 37	367 / 55	369 / 24

CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Coumaphos	210	226	306	334	362	364
	30	46	11	17	100	43
Linuron	61	124	160	162	187	248
	100	6.4	12	7.7	3.6	16
Endosulfan	195	206	241	277	339	341
	52	100	89	28	35	23
Carbophenothion	121	125	157	199	342	344
	52	37	100	29	61	29

CONFIRMATORY METHOD

H. HAZARD ANALYSIS

Refer to CHC3, Section H.

CONFIRMATORY METHOD

I. WORKSHEET

The worksheet on the following page, *Mass Spectrometry Confirmation/ Sample Information Form*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY CONFIRMATION/ SAMPLE INFORMATION FORM

(To be submitted with copy of FSIS Form 10,000-2 or FSIS Form 10,530-1)

Submitted by: _____ Date: _____

Sample I.D. Form # Lab #	UAR Approval:	Residue Code/Name 1) 2)	Violation Sample YES/NO Violation Level (ppm)

	Recovery	Tissue Blank	Sam 1	Sam 2	Sam 3	Sam 4	Sam 5
Conc. found/spiked in tissue (ppm)	1) 2)						
Absolute amount (ng) of residue in final extract (assuming 100% recovery)	1) 2)						
Tissue weight (gm) equivalent of final extract	1) 2)						

Concentration of standard submitted:

To be completed by GC/MS Operator

Final volume (μL) for SIM confirmation:

Final volume (μL) for full scan confirmation:

Approximate % recovery calculated by GC/MS:

Operator(s) _____ Start date _____

Reviewer _____ Completion date _____

CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb for all compounds.

b. No false negatives at levels listed in J.7.

2. Critical Control Points and Specifications

Refer to CHC3, section J.2.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standards, show that instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation, submitted by supervisor.

(a) A tissue blank for each type of species to be monitored.

(b) 5 samples (combination of all species), each fortified with no more than 2 types of COPs at the actionable level.

Report data to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

b. Acceptability criteria.

Refer to confirmation criteria, section F.3.

4. Intralaboratory Check Samples

a. Frequency.

i. One check sample for confirmation on a quarterly basis.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples over a period of one year.

iii. Records are to be maintained by the analyst and reviewed by the Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on difference between replicates.

CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

If unacceptable values, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify all probable causes.

iii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Fat.

b. Sample receipt size, minimum: 50 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage:

i. Time: 1 month, estimated.

ii. Condition: Frozen (– 20° C).

6. Sample Set

Each set must include a tissue blank, a tissue blank fortified with the appropriate standard at its actionable level as well as at the highest concentration found in the suspect sample, and all samples found positive from the determinative method.

7. Sensitivity

a. Lowest detectable level (LDL): not applicable.

b. Lowest reliable confirmation (LRC):

Compound	*Lowest Reliable Confirmation (ppm)*
Ronnel	0.05 (poultry), 0.05 (red meat)
Chlorpyrifos	0.05 (poultry), 0.05 (swine)
Chlorfenviphos	0.02 (poultry), 0.10 (beef)
Stirophos	0.08 (poultry), 0.10 (beef)
Phosalone	0.04 (beef)
Coumaphos	0.20 (beef)
Linuron	0.50 (beef)
Endosulfan	0.10 (beef)
Carbophenothion	0.04 (beef)

PENTACHLOROPHENOL

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 7
G. Calculations 8
H. Hazard Analysis 9
I. Worksheet 10
J. Quality Assurance Plan 13

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This procedure is based on the extraction of pentachlorophenol (PCP) into cyclohexane and removal of interfering compounds by a strong acid treatment. Quantitative transfers are eliminated by the addition of pentabromoethylbenzene (PBEB) as a reference compound. The PCP and PBEB are quantitated directly on a gas liquid chromatograph equipped with an electron capture device. The screen has a usable range from 50 to 5000 ppb.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Culture tubes, screw cap, teflon liner, 16 × 125 mm, 20 × 150 mm, and 25 × 150 mm.

b. Replacement caps for culture tubes, teflon liner, size: 15-415 and 24-410.

c. Heating blocks, Multi-Blok heaters, or equivalent, size to accept 20 mm and 25 mm tubes.

d. Reciprocating horizontal shaker, Eberbach 600, or equivalent.

e. Solvent dispensers, Brinkmann Dispensette, or equivalent, sizes 0.4 to 2 mL, 1.0 to 5.0 mL, and 5 to 25 mL.

f. Measuring syringes, sizes 100 μL, 200 μL, and 500 μL.

g. Test tube support, sizes 15-16 mm, 18-20 mm, and 22-25 mm O.D.

h. Disposable pipets, Pasteur-type, 9″ length.

2. Instrumentation

Gas liquid chromatograph, Hewlett-Packard 5840, or equivalent, equipped with Ni^{63} electron capture detector, 3′ × 2 mm id glass column packed with 1% SP-1240DA on 100-120 mesh Supelcoport.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Cyclohexane: Burdick and Jackson, or equivalent, pesticide grade.

b. 2-Propanol: Burdick and Jackson, or equivalent, pesticide grade.

c. Toluene: Burdick and Jackson, or equivalent, pesticide grade.

d. H_2SO_4: Reagent grade.

e. 12M H_2SO_4: Add 66 mL of con. H_2SO_4 to 33.0 mL H_2O.

f. Pentabromoethylbenzene: 98% pure, Aldrich Chemical Company.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

All FSIS laboratories are to procure pentachlorophenol standards from:

U. S. Environmental Protection Agency
Director, Environmental Monitoring Support Laboratory
Office of Research & Development
Cincinnati, OH 45268

2. Preparation of Standards

a. Pentachlorophenol (PCP).

i. Stock A (1 mg/mL): Weigh out a 100 $\pm$ 5 mg sample of PCP and transfer to a 100 mL volumetric flask. Dissolve sample and make up to volume with toluene.

ii. PCP working standard, range 50-500 ppb (2 μg/mL): Dilute 1 mL of stock A to 500 mL with 2-propanol, using a volumetric flask.

iii. PCP working standard, range 100-1000 ppb (4 μg/mL): Dilute 2 mL of stock A to 500 mL with 2-propanol, using a volumetric flask.

iv. PCP working standard, range 500-5000 ppb (20 μg/mL): Dilute 10 mL of stock A to 500 mL with 2-propanol, uwing a volumetric flask.

b. Pentabromoethylbenzene (PBEB).

i. Stock B (1.2 mg/mL): Weigh 120 $\pm$ 5 mg sample of PBEB and transfer to a 100 mL volumetric flask. Dissolve sample and make up to volume with toluene.

ii. Internal reference solution, 50-500 ppb (600 ng/mL): Dilute 0.5 mL of stock B to 1000 mL with 2-propanol, using a volumetric flask.

iii. Internal reference solution, 100-1000 ppb (1.20 μg/mL): Dilute 1.0 mL of stock B to 1000 mL with 2-propanol, using a volumetric flask.

iv. Internal reference solution, 500-5000 ppb (6.0 μg/mL): Dilute 5.0 mL of stock B to 1000 mL with 2-propanol, using a volumetric flask.

3. Storage Conditions

a. Stock A, Stock B, PCP working standards: Store in refrigerator.

b. PBEB internal reference solutions: Store at room temperature.

4. Shelf Life Stability

a. Stock A, Stock B: Prepare fresh every 3 months.

b. PCP working standards, PBEB internal reference solutions: Prepare fresh monthly.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Blend a 50 g liver sample in a 1-qt blender jar until a homogenous mixture is obtained. Transfer the homogenized sample to a 6″ × 4.5″ plastic bag. Heat-seal the bag and store at −20° C until assayed.

2. Sample Extraction

a. On the day of the assay, thaw the frozen liver packet to room temperature. Cut a corner from the packet and squeeze a 2.0 ± 0.1 g sample into a 150 × 25 mm glass screw cap tube, held vertical inside a beaker, on a top loading balance.

b. Using a Brinkman dispenser, add 5 mL of 12M H_2SO_4 to each tube. After each addition, immediately cap the tube with a teflon-lined cap and shake vigorously by hand to prevent large aggregates from forming. Smaller lumps on the tube walls and at the surface will be dispersed by the end of the digestion period.

c. After completing the acid additions, transfer the tubes to a heating block and heat for 60 ± 5 minutes at 97 ± 3° C. At 10-minute intervals, briefly hand-shake each tube. *Caution*: Hot H_2SO_4 is very corrosive and appropriate personal protective clothing should be worn. This includes lab coat, rubber gloves and apron, goggles, and face shield. For added protection, shake the glass tubes inside a plain, round-bottom 100 mL polypropylene centrifuge tube, behind a safety shield.

d. At the end of the heating period, cool the tubes to room temperature in a basin of water. To the cooled digestates, add 10 mL cyclohexane and 2 mL of appropriate internal reference solution, using the Brinkman dispensers. Cap tubes and shake for 15 ± 2 minutes on a mechanical shaker.

NOTE: For range 100-1000 ppb, use 20 mL of cyclohexane in step d. For range 500-5000 ppb, at the end of step d, transfer a 1 mL aliquot of the cyclohexane layer to a 16 × 125 mm glass tube and mix with 10 mL cyclohexane. Use a 4 ± 1 mL aliquot of this solution in step e.

e. At the end of the extraction period, allow phases to separate and transfer a 4 ± 1 aliquot of the upper phase to a 16 × 125 mm screw-cap glass culture tube, using a disposable glass transfer pipet.

f. Add 1 mL of concentrated H_2SO_4 to the cyclohexane aliquot, using a Brinkman dispenser. Cap the tubes with teflon-lined caps and shake on a mechanical shaker for 2 minutes.

g. At the end of the shaking period, allow the tubes to stand for 30 minutes. Transfer an aliquot of the upper phase to a gas chromato-graph autosampler vial. Cap the vials and store in a freezer. Analyze within 24 hours.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Preparation of Blank Matrix Cyclohexane

The matrix has an enhancing effect on the PCP response, necessitating the preparation of standard in a cyclohexane extract of blank liver. Prepare the extract by using the method in section E.2 with the following modifications:

a. Step E.2.a—Weigh 8.0 g.

b. Step E.2.b—Add 20 mL of 12M H_2SO_4.

c. Step E.2.d—Transfer digest to 125 mL separatory funnel; add 40 mL cyclohexane and 8.0 mL 2-propanol. Shake for 2 minutes.

d. Step E.2.e—Transfer all cyclohexane to 25 × 150 mm tube.

e. Step E.2.f—Add 5 mL concentrated H_2SO_4 and shake for 10 minutes.

f. Step E.2.g—Transfer cyclohexane to clean flask and use for preparation of GC standards.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Procedure

a. Prepare G.C. standards by mixing 10 mL of blank matrix cyclohexane (refer to section E.3); 2 mL of internal reference solution, range 50-500 ppb; and 50, 100, 200, and 500 μL of PCP working standard, range 50-500, to yield 50, 100, 200, and 500 ppb equivalent PCP in 2 g tissue respectively.

b. Chromatograph standards with samples of interest.

2. GC Conditions

a. Injector port temperature: 180° C.

b. Column temperature: 160°-180° C.

c. Detector temperature: 275° C.

d. Flow rate: 30 cc/min.

e. Sensitivity: 66 pg aldrin 50% full-scale at 2 min.

f. Auto sampler: 2 μL.

g. Column.

i. Glass: 3 ft × 2 mm glass on column injection.

ii. Packing: 1% SP-1240DA on 100/120 mesh Supelcoport.

iii. Use a very small amount of H_2PO_4 treated glass wool at the exit end only. Condition the column overnight at its maximum temperature of 189° C.

h. Retention times.

i. PCP: 2-3 min.

ii. PBEB: 4-6 min.

i. Performance.

Column should be reconditioned when peak broadening is noted or after 200 sample injections. The column can be adequately reconditioned by removing the first 6 cm of packing, scrubbing the walls with a glass wool plug, and replacing the packing with fresh material. Condition the column by elevating the temperature to 180° C overnight. The column may be hooked to the detector during this conditioning period.

3. Note

The 20-500 ppb range response curve will be the same as the 100-1000 or 500-5000 curves if the working PCP standards and internal reference solutions are equivalent after appropriate dilutions. To check the concentrations of standards in each range, add 200 μL of PCP working standard, 2 mL of 50-500, 100-1000, and 500-5000 ppb ranges respectively. The GC quantitation of these solutions, expressed as the ratio of PCP to PBEB, should vary by less than 5%. Care needs to be taken to adjust the Brinkman dispensette so that the same volume of internal reference solution is delivered for all ranges.

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Measure the peak heights of PCP and PBEB. Calculate the ratio of PCP to PBEB.

$$y = \frac{\text{height of PCP peak}}{\text{height of PBEB peak}}$$

b. Using a linear regression, construct a standard curve of peak height ratio vs. equivalent ppb PCP.

The equation is $y = mx + b$.

Where: y = ratio peak height PCP to peak height PBEB.
x = ppb PCP.
m = slope.
b = y-intercept.

The correlation coefficient (r) should be ≥ 0.990.

c. Using the regression slope and intercept, the PCP concentration for each incurred sample may be calculated.

d. PCP determination.

i. Measure peak height of PCP and PBEB and express as the ratio of PCP to PBEB.

ii. Determine the amount of PCP by substituting the PCP/PBEB ratio into the standard curve equation

$y = mx + b$.

Where: x = PCP in ppb.
m = slope of standard curve.
y = PCP/PBEB peak height ratio.
b = intercept of standard curve.

iii. Correct for recovery by dividing the calculated amount of PCP in step d.ii by the % recovery times 100.

e. Recovery.

i. With each set, run 2 blank tissues fortified with 100 mL of PCP working standard of appropriate range.

ii. Calculate the amount recovered using the equation in step d.ii.

iii. Percent recovery is determined by dividing the fortification level into the amount recovered.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Screening Procedure for Pentachlorophenol in Liver Tissues

2. Required Protective Equipment

Safety glasses, face shield, heat-resistant gloves, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
E.2.c, d.		
After completing acid additions (12M H_2SO_4), transfer the tubes to a heating block and heat for 60 ± 5 min at 97° C ± 3° C.	Acid spattering and corrosive vapors causing skin, eye, and respiratory damage. Explosion.	In addition to the caution statement given in this step, the analyst should work in a fume hood. The heating block temperature should be monitored closely in order to reduce the explosion risk.
Cyclohexane	Flammable. Skin and respiratory irritant.	Perform this addition in an efficient fume hood.

4. Disposal Procedures

Acidic aqueous waste	See above	Partially neutralize with 50% w/v NaOH and flush into waste disposal tank.
Cyclohexane	See above	Store in nonchlorinated waste container until disposed of by contractor or in-house specialist.

DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *PCP*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

PCP WORKSHEET

Set # _____ Date _____

PCP Working Standard: Range _____ Date Prepared _____

Inter Reference Solution: Range _____ Date Prepared _____

TUBE NUMBER	PEAK HEIGHT		RATIO PCP/PBEB*	PCP (ppb, uncorrected)
	PCP	PBEB*		
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25 (Fortified)				
26 (Fortified)				
STD 50				
STD 100				
STD 200				
STD 500				

Slope = _____ Correlation coefficient = _____

Intercept = _____ Recovery = _____

*PBEB = Pentabromoethylbenzene (Internal standard)

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability %CV*	*Reproducibility %CV*
PCP	30-5000	85-110	≤ 15	≤ 20

2. Critical Control Points and Specifications

a. Sample weight. 2.00 g ± 0.05 g.

b. Temperature of digestion block. 97° C ± 3° C.

c. Digestion time. 60 min ± 5 min.

d. Volume internal standard. 2.00 mL ± 0.02 mL.

e. GC column performance. A symmetrical peak.

f. Peak height, ratio, PCB:PBEB correlation coefficient.† r > 0.990

g. Recovery of fortified samples.† 85-110%

†A written record must be kept.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—On 3 consecutive days, prepare the GC standard curve as described in the method at 50, 100, 200, and 500 ppb.

ii. Phase II:Fortified samples—On 3 consecutive days, perform 3 sets of recoveries. Each set should consist of 6 blank liver samples (any species) fortified at 50, 100, 200, and 500 ppb.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Check samples for analyst accreditation.

(a) 14 samples submitted by FSIS Science & Technology Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report analytical findings to Chemistry Division, QSB.

b. Acceptability criteria.

Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Initially, minimum of 1 biweekly per analyst.

ii. Random replicates or blind samples chosen by supervisor or Laboratory QA Officer.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Records to be maintained by analyst and reviewed by supervisor Laboratory QA Officer.

(a) All replicate findings.

(b) CUSUM charts.

(c) All percent recoveries recorded.

(d) Running average, standard deviation, and CV for all recoveries.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrices: Liver, muscle.

b. Sample receipt size: Varied; enough to obtain matrix for all required quantitative and confirmation tests and reserve sample.

c. Condition upon receipt: Cold.

d. Sample storage:

i. Time: Up to 6 months.

ii. Condition: Frozen.

6. Sensitivity

a. Lowest detectable level (LDL): 25 ppb.

b. Lowest reliable quantitation (LRQ): 50 ppb.

c. Minimum proficiency level (MPL): 50 ppb.

SYNTHETIC PYRETHRINS

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 8
G. Calculations 12
H. Hazard Analysis 13
I. Worksheet 14
J. Quality Assurance Plan 17

II. Confirmatory Method

A. Introduction 20
B. Equipment 20
C. Reagents 20
D. Standards 20
E. Derivatization 20
F. Analytical Confirmation Procedure 21
G. [Reserved]
H. Hazard Analysis 26
I. Worksheet 26
J. Quality Assurance Plan 29

I. DETERMINATIVE METHOD

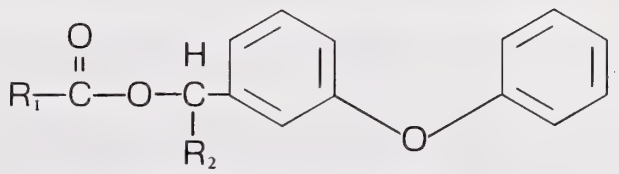
A. INTRODUCTION

1. Theory and Structure

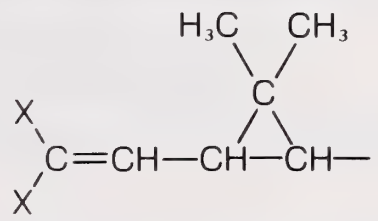
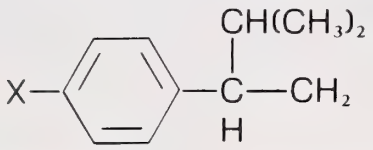
Synthetic pyrethrins are used for their insecticidal action.

In this method, samples are extracted and partitioned using a modified version of the chlorinated hydrocarbon method. Florisil column chromatography is used to purify the sample extracts, using mixtures of hexane-toluene to separate pyrethrins from GC-interfering compounds. Residues in the eluate are measured quantitatively using capillary gas chromatography and may be confirmed using capillary gas chromatography/mass spectrometry.

Structures of the synthetic pyrethrins analyzed by this procedure are similar, since pyrethroid activity is structure dependent. The general structure for all the compounds is:



Structures unique to each compound are summarized below:

Compound	*Mol. Wt.*	R_1	*X*	R_2
Permethrin	391.3		Cl	TH
Cypermethrin	415.3		Cl	CN
Deltamethrin	505.2		Br	CN
Fenvalerate	419.9		Cl	CN
Flucythrinate	451.4		OCF_2H	CN

2. Applicability

This method is applicable to bovine and poultry fat.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Equivalent apparatus and instrumentation may be substituted for those specified unless otherwise stated.

a. Chromatographic columns: 10.5 mm id × 25 cm with 200 mL reservoir (Kontes K-420280-0213).

b. Filter tube: 10 mL glass syringe body, or any tube with an id of about 1.5 cm and length of 10 cm or more. Use glass wool plug.

c. Kuderna-Danish concentrators: 250 mL with 4 mL graduated tube (Kontes K-570025).

d. Separatory funnels: 60 and 250 mL, with glass stoppers and Teflon stopcocks.

e. Concentrator: Organomation N-Evap or equivalent device for evaporation by nitrogen flow and heating.

f. Centrifuge tubes: Glass, 15 mL graduated tubes with stopper.

g. Three-ball Snyder columns.

2. Instrumentation

a. Gas Chromatograph: Hewlett-Packard 5890A or equivalent, equipped with electron capture detector, 1 mv recorder, and appropriate adapters (injection port insert and make-up gas) to allow use of 0.53 mm capillary column.

b. Column: DB-608 capillary, 15 or 30 m × 0.43 mm id, 1 μm film thickness (J&W Scientific or equivalent).

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

NOTE: All solvents used should be certified for use in pesticide analysis. If reagent blanks contain interferences, solvents should be concentrated in proportion to their use in the procedure to test for the source of interference.

a. Petroleum ether: Burdick & Jackson high purity, or equivalent.

b. Acetonitrile: Burdick & Jackson high purity, or equivalent.

c. Hexane: Burdick & Jackson high purity, or equivalent.

d. Acetone: Burdick & Jackson high purity, or equivalent.

e. Toluene: Burdick & Jackson high purity, or equivalent.

f. Sodium sulfate, anhydrous, granular, reagent grade.

g. Florisil: Pesticide (PR) grade, activated, 60/100 mesh.

Dry 48 hours at 140° C. Cool to room temperature in sealed container, then deactivate by adding approximately 5% water, reseal, and mix by shaking until no lumps remain. Allow to equilibrate 3 hr or more before use. Store in a sealed container in the dark. Deactivated florisil is stable for at least a week.

h. Acetonitrile saturated with petroleum ether: Saturate acertonitrile with petroleum ether. A small layer of petroleum ether should be visible on the acetonitrile.

i. Acetone/hexane, 5% v/v.

j. Eluting solvent, 10% toluene in hexane: Dilute 100 mL of toluene to 1 L with hexane.

k. Sodium sulfate solution, 4% (w/v). Place 40 g of sodium sulfate in a clean 1 L volumetric flask and dilute to volume with distilled water.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Cis-permethrin, trans-permethrin, cypermethrin, flucythrinate, fenvalerate, and deltamethrin may be obtained from:

Director, Environmental Monitoring Support Laboratory
Office of Research and Development
Cincinnati, OH 45268

2. Preparation of Standards

NOTE: Cypermethrin and flucythrinate cannot be combined in a mixed standard because some of their peaks co-elute.

a. Individual stock standards (1 mg/mL).

Accurately weigh 25.0 mg of each pyrethrin reference standard into a clean 25 mL volumetric flask and dilute to volume with 5% acetone/hexane.

b. Intermediate mixed standard (10 μg/mL).

Pipet 1.0 mL of each stock standard to be included in mixed standard into a clean 100 mL volumetric flask and dilute to volume with hexane.

c. Fortification standards (1 μg/mL).

Pipet 1.0 mL of intermediate mixed standard solution into a clean 10 mL volumetric flask and dilute to volume with hexane. Use for fortification at 1 ppm level.

d. Fortification/gas chromatography standards (100 ng/mL).

Pipet 1.0 mL of intermediate mixed standard solution into a clean 10 mL volumetric flask and dilute to volume with hexane. Use for fortification at 100 ppb level and as external standard for GC quantitation.

e. Fortification/gas chromatography standards (25, 50, 75 ng/mL).

Pipet 2, 4, and 6 mL aliquots of 100 ng/mL standard into 10 mL volumetric flasks. Pipet 6, 4, and 2 mL aliquots of hexane to the three flasks so the final volume in all three is 8 mL. Use for fortification at 25, 50, and 75 ppb levels respectively.

3. Storage Conditions

Store all standards in a freezer at 0° C or less.

4. Shelf Life Stability

Actual shelf life of standards stored under conditions described is not known. Stock and intermediate standards have shown no deterioration after more than a year of storage under these conditions, and may be stable indefinitely. Standards at concentrations of 1 μg/mL or less are also quite stable, showing no deterioration after 2-3 months of storage. Longer-term studies of dilute solutions have not been made. A recommended procedure is to make up new working standards every month or whenever a change in the relative peak height of one or more of their components is observed.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Pass chilled sample through a food chopper twice. Plug a powder funnel with glass wool. Put approximately 100 g of the comminuted fat in the funnel, place the funnel in a beaker, and render at 100-110° C until fat ceases to drop.

2. Sample Extraction

a. Weigh 1 g of the melted fat into a 25 mL beaker, and transfer contents to a 60 mL separatory funnel using a total of 10 mL of petroleum ether to effect the transfer. Alternatively, 1 g of liquid fat can be added to a tared separatory funnel containing 10 mL of petroleum ether.

b. In order to check for recovery, fortify a sample of blank fat prepared as in step a above with 1.0 mL of 50 or 100 ng/mL fortification standard to provide a 50 or 100 ppb sample.

c. Add 10 mL of acetonitrile saturated with petroleum ether to the 60 mL separatory funnel and shake vigorously for 1 min. Allow layers to separate completely.

d. Drain the bottom layer into a 250 mL separatory funnel containing 100 mL of 4% sodium sulfate solution and 30 mL petroleum ether.

e. Repeat steps b and c twice more, collecting all extracts in the 250 mL separatory funnel.

f. Stopper and invert the 250 mL separatory funnel, vent pressure, and shake gently for 1 min to transfer pesticides to the petroleum ether.

g. Let layers separate cleanly, then drain and discard the aqueous layer.

h. Wash the petroleum ether in the 250 mL separatory funnel with two 15 mL portions of 4% sodium sulfate. Stopper, invert, and vent after each addition. Discard washings.

i. Draw off petroleum ether through a 3 cm layer of sodium sulfate in a filter tube into a 125 mL Kuderna-Danish concentrator flask equipped with a 5 mL concentrator tube.

j. Rinse the separatory funnel with 10 mL of petroleum ether and drain through the sodium sulfate column into the flask.

k. Attach a 3-ball Snyder column to the flask and evaporate the petroleum ether extract to <4 mL, using a hot water or steam bath.

l. Pack a small wad of glass wool (<5 mm high) into the bottom of the chromatography column. Add 2 g florisil (as prepared in section C, Reagent and Solution List, item g), then top with 1 cm of sodium sulfate. Prewash the column with 10 mL of 10% toluene/hexane and discard the wash.

m. Transfer the petroleum ether extract in the concentrator tube to the column, draining at a rate not to exceed 1 mL/min. Allow the liquid to drain to the top of the florisil layer. Discard the eluate.

n. Rinse the concentrator tube with 3 mL of petroleum ether and add it to the column. Drain rinse until the liquid level is at the top of the florisil layer. Discard the eluate.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

o. Elute column with 15 mL of 10% toluene in hexane at less than 1 mL/min. Discard the eluate.

p. When the last of the wash eluate has drained completely from the column, place a 15 mL centrifuge tube under the column and elute with a sufficient volume of toluene to remove the compounds of interest. (See section E.3 below for determination of appropriate elution volumes.)

q. Concentrate the toluene eluate to 50-100 μL on an N-evap using dry nitrogen and a water bath temperature of 35-40° C. Reconstitue the residue to an appropriate volume with hexane (1 mL for quantitation at 50 ppb level, 10 mL at 1 ppm level.)

3. Standardization of Florisil

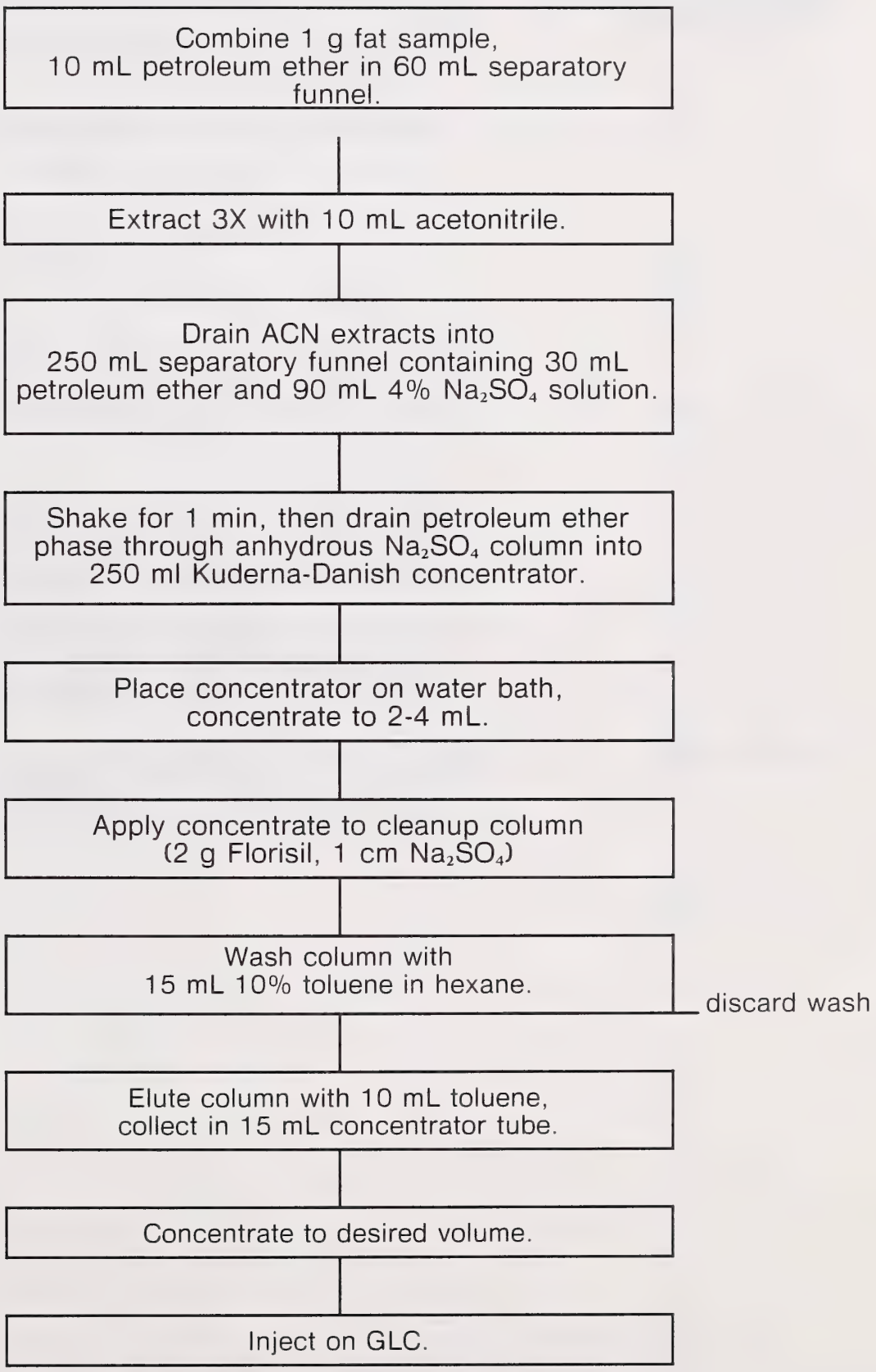
In order to minimize analyst time and solvent usage required for the cleanup, dimensions of the cleanup column have been kept as small as possible. Because the efficency of this column is low, it is important to calibrate elution volumes when a new lot of florisil is used. Interfering substances elute from the cleanup just after the fraction containing synthetic pyrethrins. If too much eluant is collected, late-eluting interferences may make quantitation of low levels of permethrin and cypermethrin difficult. If too little eluant is collected, incomplete elution of the polar synthetic pyrethrins will occur, resulting in low recoveries for those compounds.

Test each new lot of florisil as follows: Using the procedure described in section E.2, extract and partition a blank fat sample, fortifying the concentrated extract with a mixed standard (100 ppb level) just prior to florisil column chromatography. Prepare column as described in section E.2.k above. Apply fortified blank wash with 10% toluene/hexane. Elute pyrethrins with 12 mL toluene, collecting aliquots of 7, 1, 1, 1, 1, and 1 mL. Inject aliquots on a gas chromatograph and record the volumes at which: a) greater than 95% of cis- and trans-permethrin have elutes and b) greater than 95% of all remaining compounds of interest have eluted. Typically, the permethrins should elute in the first 7 mL aliquot, and the remainder should have eluted within a volume of 9-10 mL. (Later aliquots will contain increasing amounts of interfering compounds.) If more than 10 mL of toluene is required to elute all pyrethrins, the level of deactivation may be increased in 0.5% increments until all compounds elute with 9-10 mL.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

a. GC operating conditions:

i. Carrier gas: Helium at 6 mL/min (15M column) or 10 mL/min (30M column).

ii. Make-up gas: 5% methane/argon at 25-30 mL/min.

iii. Injector temperature: 230° C.

iv. Column temperature: 250-255° C.

v. Detector temperature: 300° C.

b. Adjust the electrometer and recorder parameters so that 0.1 ng of the compound of interest (1 μL of 100 ng/mL std.) produces at least 70% full-scale deflection for the largest peak in the standard. Baseline noise should be less than 1% full scale.

c. Before attempting routine analysis, verify system linearity by injecting standards at 25, 50, 75, and 100 ng/μL. (Verification of detector linearity should be done at least monthly, or whenever there is reason to suspect degradation of system performance.)

d. Inject a 1 μL aliquot of the eluate into the chromatograph. If the peaks are offscale or out of linear range, dilute the sample to bring response into the linear range. Tentatively identify peaks on the basis of their retention time.

2. Interferences

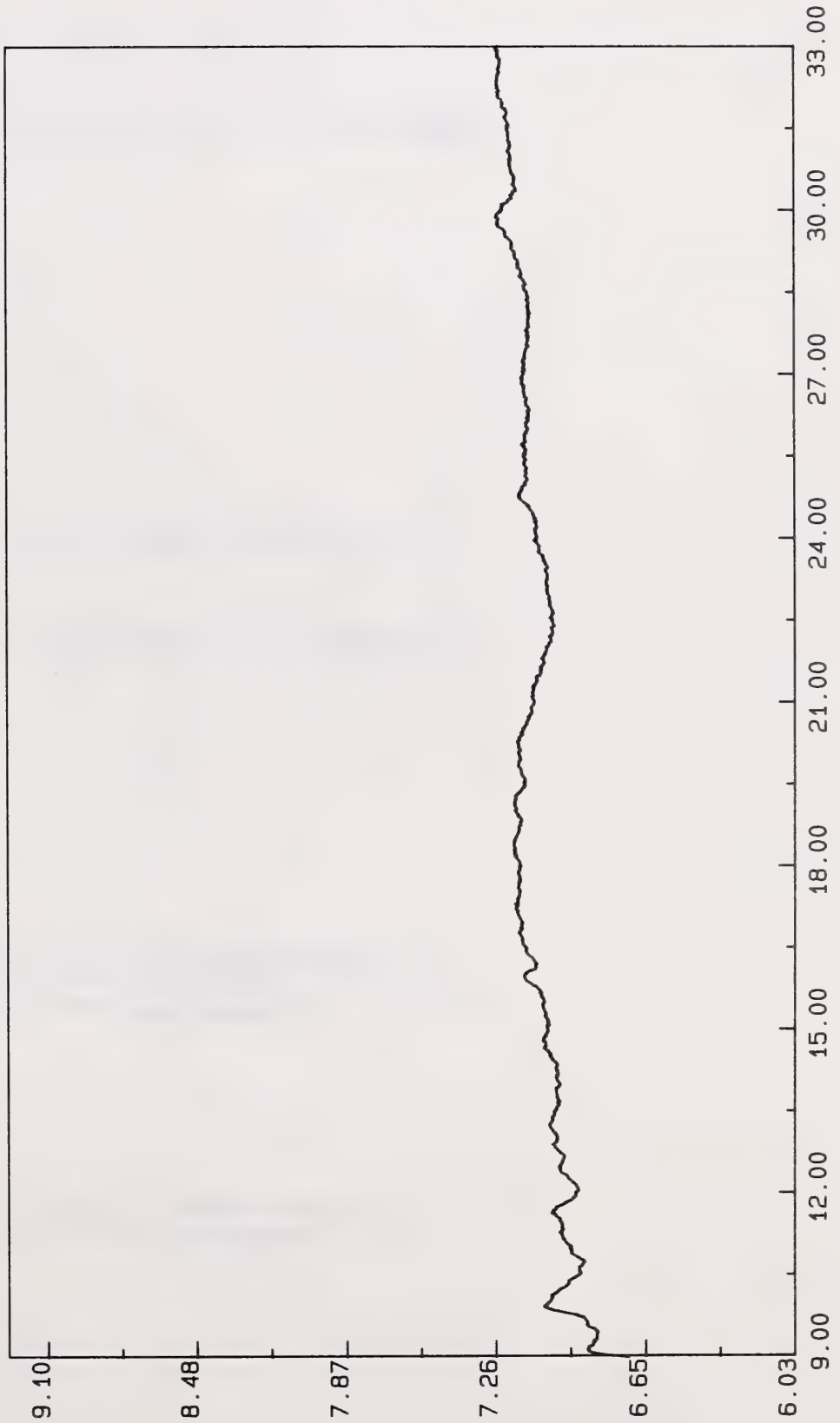
Some fat samples contain peaks that coelute with permethrin, particulary the cis isomer. This can make quantitation difficult when low concentrations (25 ppb or less) of these compounds are present.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. Sample Chromatograms

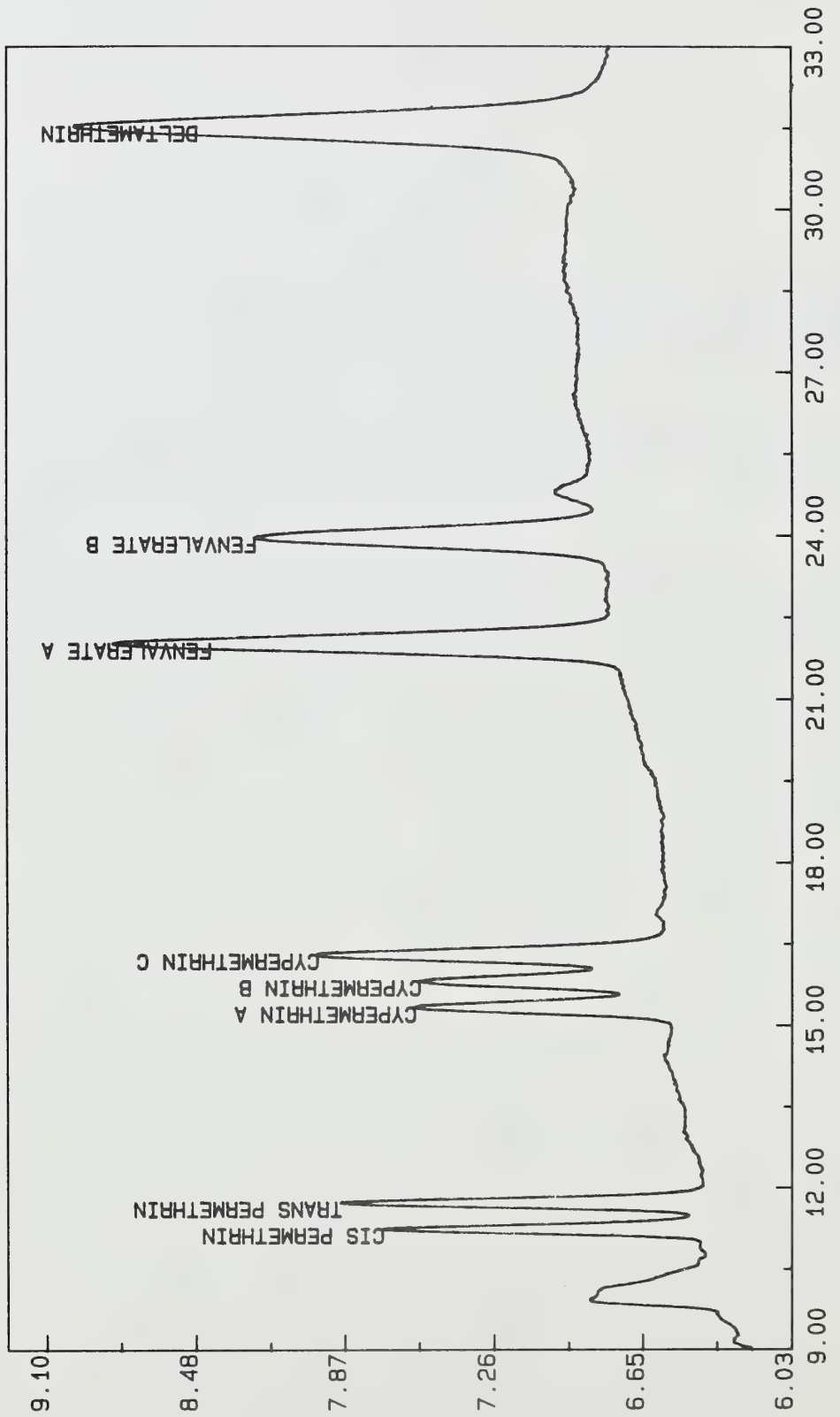
Synthetic Pyrethrins Tissue Blank.



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

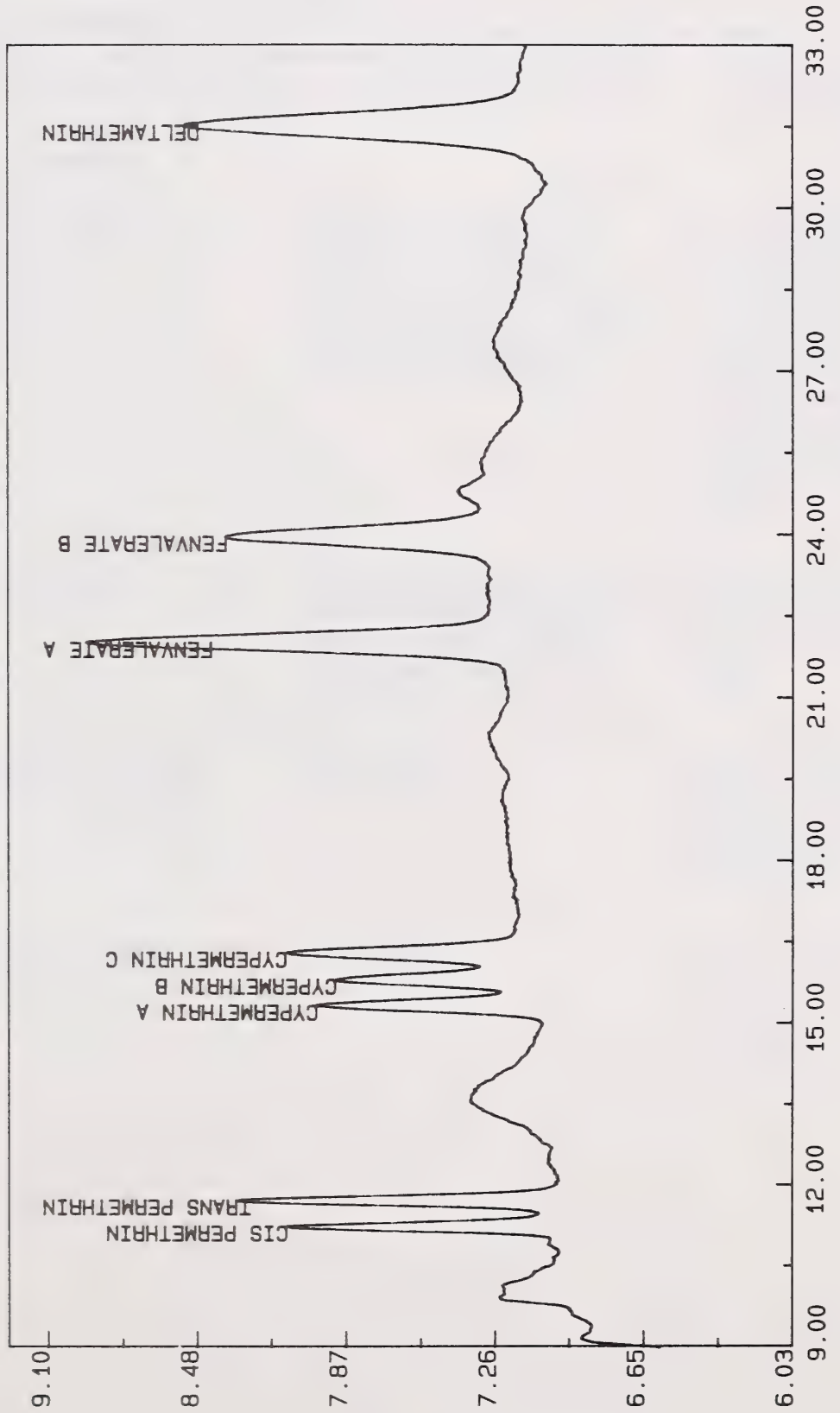
Ext. 50 ppb—Synthetic Pyrethrins.



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

Rec. 50 ppb—Synthetic Pyrethrins.



I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

By an acceptable means, measure the peak areas, or peak heights for each component in the mixed standard. Calculate the concentration of pyrethrin present using the formula:

$$\text{Conc. (ppm)} = \frac{\text{peak ht/peak area (sample)} \times \mu\text{L injected (stnd)} \times \text{conc. stnd } (\mu\text{g/mL}) \times \text{vol. final ext. (mL)}}{\text{peak hgt/peak area (stnd)} \times \mu\text{L injected (sample)} \times \text{weight of sample (g)}}$$

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Analysis of Synthetic Pyrethrins by Gas Chromatography

2. Required Protective Equipment

Safety glasses, protective gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Petroleum ether Acetonitrile Hexane Toluene	These solvents are all flammable and can produce toxic effects through exposure to the skin, eyes, respiratory system, or ingestion.	All procedures involving solvents should be carried out in an efficient fume hood and away from any source of ignition. Protective gear should be worn for all steps where the possibility of solvent spillage exists.
D. Standards	Concentrated standards may be toxic. Exposure paths are the same as described above for reagents.	Prepare standards in a fume hood or glove box. Use protective gear.

4. Disposal Procedures

Reagent mixtures used in section E.	See above.	Hold in designated storage cans awaiting disposal by the contractor or in-house specialist
Florisil columns (used)	See above.	Allow columns to dry in a fume hood. Dispose of packing by pouring into a plastic bag and discarding.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Pyrethrins Analysis,* can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

Worksheet for Pyrethrins Analysis

Analyst _____

Date _____

Sample	Peak Heights									
	Permethrin		Cypermethrin			Fenvalerate		Flucythrinate		Delatamethrin
	cis	trans	A	B	C	A	B	A	B	
Standard 1, ppb:										
Standard 2, ppb:										
Recovery ppb:										
CheckSample #										
Blank #										

Calculated results

Sample	Amount Found					
	Permethrin		Cypermethrin (Total)	Fenvalerate (Total)	Flucythrinate (Total)	Delatamethrin
	cis	trans				
Recovery #						
Check Sample #						

Parameters: Column: 15 M x 0.53 mm x 1.0 u DB-608; Detector: EC

Operating Conditions: Carrier: He at 6 ml/min; Make-up gas: 5% CH3/Argon @ 30 ml/min

Temperatures: Inlet - 230; Column - 255; Detector - 300

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppb)*	*Acceptable Recovery (%)*	*Repeatability CV (%)*
Cis-permethrin	10-100	70-110	<20
Trans-permethrin	10-100	70-100	<20
Cypermethrin	10-100	80-110	<20
Flucythrinate	10-100	80-110	<20
Fenvalerate	10-100	80-110	<20
Deltamethrin	10-100	80-110	<20

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Solvent lot number, date checked, findings	No interfering peaks with deflection >2% full scale in reagent blank when 100 ppb standard gives >50% response.
b.	Sample weight	1.00 ± 0.02 grams.
c.	Time and temperature for Florisil drying	Dry at least 24 hours at 135-140° C before using.
d.	Amount of deactivation to achieve desired recoveries	All compounds with >95% recovery through column with 10 mL toluene elution.
e.	Column elution rate	Less than 1.0 mL/minute for all elution steps.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Analyze standards at following concentrations, with three replicates for each compound (hand injected or by auto-injection, whichever way the method will be routinely performed.).

25, 50, and 100 ppb for cis- and trans-permethrin, cypermethrin, fenvalerate, and deltamethrin (mixed standard). 25, 50, and 100 ppb for flucythrinate (separate standard).

ii. Phase II: Recoveries—Fortified samples must include at least three levels with three replicates each, for each compound of interest. Each set sould include a reagent blank and tissue blank. Analyze concentrations at:

Sample	*Tissue Concentrations*
Poultry Fat	
Cis-permethrin	25, 50, 100 ppb
Trans-permethrin	25, 50, 100 ppb
Cypermethrin	25, 50, 100 ppb
Fenvalerate	25, 50, 100 ppb
Deltamethrin	25, 50, 100 ppb
Bovine Fat	
Cis-permethrin	1, 2, 4 ppm
Trans-permethrin	1, 2, 4 ppm
Flucythrinate	1, 2, 4 ppm
Cypermethrin	25, 50, 100 ppb
Fenvalerate	25, 50, 100 ppb
Deltamethrin	25, 50, 100 ppb

iii. Phase III: Check samples submitted by laboratory supervisor or LSO. Each sample must randomly appear at least three times per species at concentrations within the range used in phase II. The number of analytes per sample should be varied. Report all analytical findings, through laboratory LSO, to the Chemistry Divison as follows:

Phase I and II for information only, the acceptability of this data to be determined by the laboratory supervisor and the LSO.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

i. Refer to section J.1 above.

ii. No false positives.

iii. No pyrethrins missed if present at or above the lowest detectable limit, which is 10 ppb for all pyrethrins.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst.

ii. Blind check samples or random duplicates chosen by the supervisor or Laboratory QA Officer.

iii. Records to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer:

(a) All duplicate findings.

(b) All recovery values.

(c) Running average, standard deviation, and CV for all recoveries.

(d) CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Fat

b. Sample receipt size: Sufficient tissure for all quantative and confirmational analyses and sample reserve portion (approximately 200 g).

c. Condition on receipt: Chilled to frozen.

d. Sample storage:

i. Time: 3 months.

ii. Condition: –4° C or below until analyzed.

6. Sample Set

a. A blank tissue.

b. Fortified blank at 50-100 ppb.

c. Samples.

7. Sensitivity

a. Lowest detectable level (LDL): 10 ppb.

b. Lowest reliable quantitation (LRQ): 20 ppb.

II. CONFIRMATORY METHOD

A. INTRODUCTION

Refer to Determinative Method, section A.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.1.

2. Instrumentation

GC/MS: Hewlett-Packard 5890 gas chromatograph with split/splitless capillary inlet, interfaced via direct capillary inlet to a Hewlett-Packard 5970 Mass Selective Detector.

C. REAGENTS AND SOLUTIONS

All reagents and solutions as in Determinative Method, section C.

D. STANDARDS

Use 1 μg/mL or 100 ng/mL spiking standards prepared in Determinative Method, section D.2.b.1.

E. DERIVATIZATION

Sample Preparation

a. Select two blank samples from the set analyzed. Fortify one of them at a level equivalent to that found in the confirmation sample, using the 1 μg/mL spiking sample.

b. Concentrate the confirmation sample and fortified blank on an N-Evap. Volume should be such that the concentration of the analyte of interest is 0.5-1.0 μg/mL or more. (At 50 ppb, volume would be 50-100 μL).

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC conditions and parameters.

i. Column: Hewlett-Packard Ultra 1 (bonded methyl silicone), 12 m × 0.2 mm id × 0.33 μm.

ii. Injection mode: Grob splitless.

iii. Temperature programming: 220° C.

Interface temperature: 265° C.

iv. Oven temperature: Hold 85°C 1 min. Ramp to 265° C at 20°/min. Hold at 265° C for 10 min.

v. Carrier: Helium at 2.5 psig head pressure.

vi. Injector purge delay: 0.7 min.

b. MS SIM Parameters

i. Start time: 10.0 min.

ii. Electron multiplier voltage: Autotune voltage + 200.

iii. SIM dwell times: 70 milliseconds/ion

iv. Monitored ions: See Table I.

v. Stop time: 17.0 min.

c. General requirements: The GC/MS system should be in good operating condition and the analyzer should be tuned just prior to starting a confirmation series. Use of a low-resolution mode is acceptable.

2. Required Samples for GC/MS Analysis

a. Inject 1 μL of the the 1 μg/mL spiking standard containing the compounds of interest. Verify that system response is adequate by observing ion chromatogram of one of the least intense ions monitored (See Table 1 below.)

b. If response of the standard is satisfactory, inject 1 μL each of the blank, the fortified blank, and the confirmation sample to complete the confirmation set.

c. Using the standard, determine retention times and relative ion ratios for all injections. Ratio all ions to the base peak as defined in Table 1 below. Ratios determined for the sample should be compared to both the external standard and fortified blank for best match.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

3. Criteria for Confirmation

a. Retention time specification:

Retention time of the sample peak(s) should match that of the standard or fortified blank within 2%.

b. Ion abundance ratio specification (see Table 1 for ions).

i. Ion chromatograms for at least 4 of the 6 monitored ions should contain peaks at the appropriate retention time.

ii. Ratios of at least 2 ions relative to the base peak should match those of the standard within 15%.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

TABLE 1. Mass Spectrometric Confirmation Parameters

Compound	*Rt. Time*	*Monitored Ions (See note below.)*			
Permethrin, cis	12.1	163(20) 183(100)	165(17) 255(3-4)	184 (13) 390†(2-3)	127‡
Permethrin, trans	12.2	163(25) 183(100)	165(21) 255(3)	184 (13) 390†(2)	127‡
Cypermethrin A	13.1	163(100) 181(90)	165(65) 208(37)	180 (25) 209 (35)	127‡
Cypermethrin B	13.2	163(100) 181(72)	165(66) 208(21)	180 (22) 209 (27)	127‡
Flucythrinate A	13.4	199(100) 209(18)	157(70) 225(40)	181 (31) 451 (62)	107‡
Cypermethrin C	13.4	163(100) 181(77)	165(65) 208(30)	180 (31) 209 (30)	127‡
Flucythrinate B	14.0	199(100) 209(15)	157(75) 225(33)	181 (30) 451†(61)	107‡
Fenvalerate A	14.5	167(100) 181(60)	169(45) 209(20)	225 (60) 419†(30)	152‡
Fenvalerate B	14.9	167(100) 181(60)	169(40) 209(25)	225 (55) 419†(30)	152‡
Deltamethrin	15.9	181(100) 251(38)	208(25) 253(75)	209 (27) 255 (36)	172‡

Numbers in parentheses represent typical relative ion intensities, normalized to 100. These values will vary depending on the state of tune of the mass analyzer used.

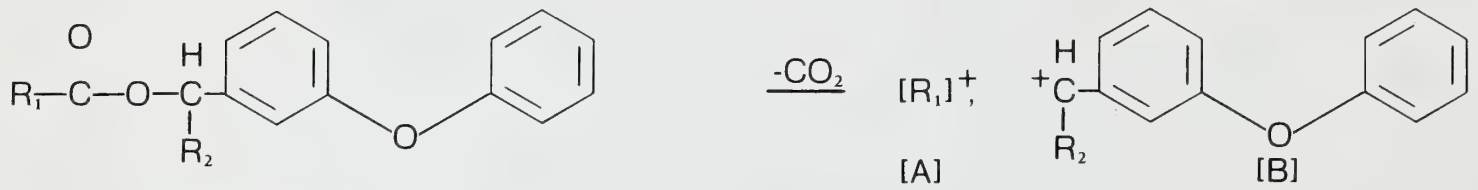
† Molecular ion

‡ Additional ion to monitor, if one of others is subject to serious interference.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

Synthetic pyrethrins appear to undergo initial EI fragmentation in the following manner:



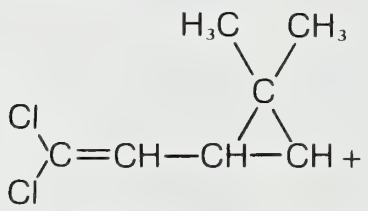
For permethrins, R_2 = H; for all other compounds, R_2 = CN. R_1 varies with each compound. (See below.)

For permethrins, Fragment [B] is the ion $(C_{13}H_{11}O)^+$, m/e 183. It appears to be a stable ion that does not yield additional useful fragments.

For all other compounds, fragment [B] is the ion $(C_{14}H_{10}NO)^+$, m/e 181.

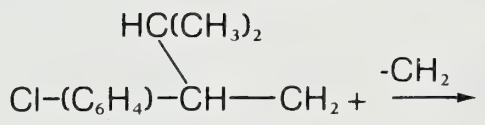
The fragment [A] is different for each compound. Suggested identities are shown below.

Permethrin, Cypermethrin



[163]

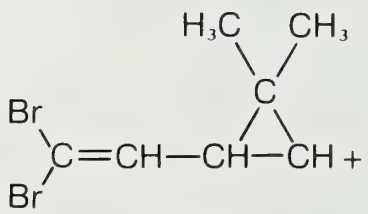
Fenvalerate



[167]

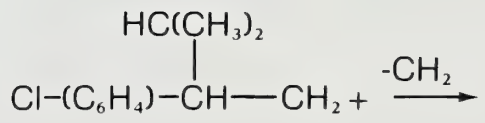
[181]

Deltamethrin



[253]

Flucythrinate



[199]

[213]

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

4. Interferences	Interferences for certain monitored ions may occur, depending on the nature of the sample and the efficiency of the cleanup. They are more serious with ions of lower molecular weight. If interferences make confirmation impossible, the extract should be taken through column cleanup a second time, using solution parameters optimized for that compound.

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

Refer to Determinative Method, section H.

I. WORKSHEET

The worksheet on the facing page, *Mass Spectrometry Data Form,* can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte: _____ Tissue: _____

	R.T. (min) Base Peak	Ion Abundance (m/z) _____	_____	_____	_____	Ion Ratios _____	_____	_____
Blank								
Standard								
Fortified Tissue								
Sample #								

Concentration:

Standard: _____

Fortified tissue: _____

Sample: _____

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards	Analyst must be able to successfully confirm the compounds of interest at a 50 ppb level.
2. Critical Control Points and Specificiatons	a. GC parameters. System must be capable of resolving all synthetic pyrethrins with good peak shape and no significant losses of analyte injected. b. MS parameters. Instrument should be tuned prior to running confirmation set. It should exhibit sufficient sensitivity so that injection of a standard containing 0.5-1.0 ng of the compound of interest will produce acceptable ion chromatograms for all ions monitored.
3. Readiness to Perform	a. Familiarization. The analyst should perform three confirmation analyses, each on a separate day, for each compound of interest at its action level, or at 50 ppb in absence of a published action level. This procedure should be repeated for each tissue to be analyzed. b. Acceptability criteria. All confirmation data must meet the standards specified for confirmation in Confirmatory Method, section F.3.a,b.
4. Check Samples	Confirmation procedure is not quantitative. Confirmation set contains all necessary QC elements for routine operation.
5. Sample Acceptability and Stability	Any sample extract that provides an acceptable clean chromatogram when analyzed using the GC/ECD as described in Determinative Method, section F, is likely to produce an acceptable confirmation. Extracts should be stored in a freezer at 0° C or less pending confirmatory analysis. Extracts should be stable indefinitely. Acceptability of stored extracts can be verified by reinjection on a GC/ECD instrument.
6. Set Construction	A confirmation set consists of an external standard at a concentration similar to that of the confirmation sample, the sample itself, a blank fortified at a level similar to that found in the sample, and a blank.
7. Sensitivity	a. Lowest detectable level (LDL): This cannot be accurately specified, since it is a function of the cleanliness of the sample extract. b. Lowest reliable confirmation (LRC): Confirmation at a 50 ppb level should be possible for all compounds. Levels significantly lower than this may or may not be confirmable, depending on the cleanliness of the sample extract.

SYNTHETIC PYRETHRINS/C-ELISA

Contents

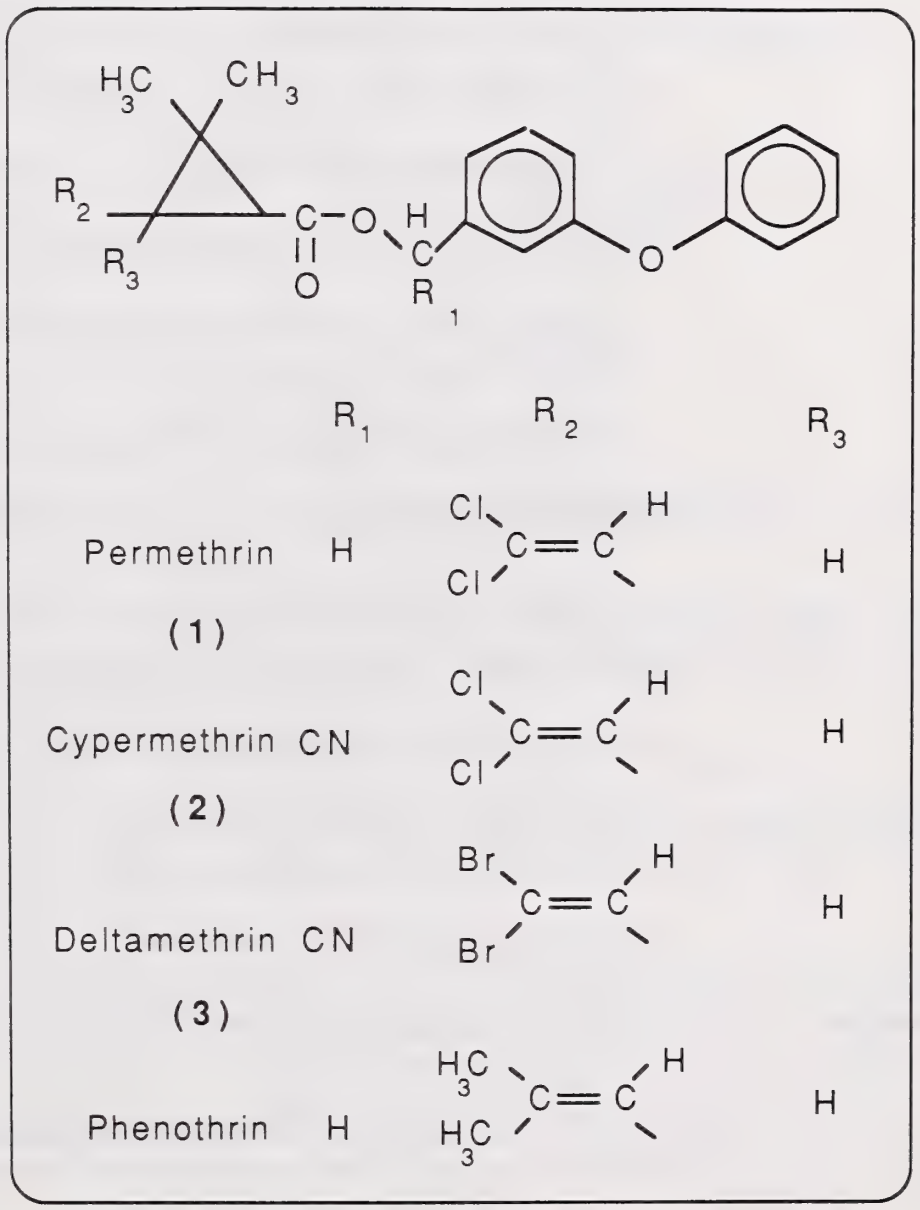
A. Introduction 1
B. Equipment 2
C. Reagents 3
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 9
G. Calculations 12
H. Hazard Analysis 17
I. [Reserved]
J. Quality Assurance Plan 18

DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structure

Rendered fat is extracted with a mixture of acetonitrile/water, centrifuged, and chilled. The acetonitrile phase is diluted with 2% NaCl and the synthetic pyrethrins are partitioned into hexane. A portion of the hexane extract is further purified using alumina column chromatography. The column eluate is concentrated, adjusted to 6% acetonitrile in phosphate-buffered saline, and analyzed with a competition enzyme-linked immunosorbent assay (c-ELISA).



2. Applicability

This method is applicable to bovine, porcine, and poultry fat. Synthetic pyrethrins are used for their insecticidal action. The monoclonal antibody used in this assay will detect the following pyrethroid insecticides: permethrin, cypermethrin, deltamethrin, phenothrin, and fenpropathrin. Flucythrinate and fenvalerate are only weakly detected, and tetramethrin is not recognized by the antibody. Refer to section J.7 for sensitivities to various pesticides. Identification and quantitation of the individual pyrethroids detected is not possible, because ELISA response is integrated over the spectrum of all detectable compounds.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

a. Centrifuge (Sorval T6000B with adapter for 50 mL tubes).

b. Centrifuge tubes, 50 mL polyproylene disposable, with cap (Beckton-Dickinson Falcon 2098).

c. Water bath, heated circulating (Freas model 260).

d. 125 mL glass separatory funnel with stopper.

e. Centrifuge tube, glass, 15 mL graduated, with glass stopper.

f. Pasteur pipet, disposable, 5.25″ (Scientific Products, Cat. P5202-1).

g. Pipetter, 50-200 μL adjustable, 8-channel, with tips (Titertek digital multichannel pipette, Flow Laboratories).

h. Pipetters, 2-20, 10-100, and 100-1000 μL adjustable single-channel, with tips (Gibson Pipetman P-20, P-100, P-1000).

i. Immulon II 96-well microtiter plates (Dynatech Laboratories).

j. Sealing tape, 8.26 × 13.34 cm, for microtiter plates (Dynatech Laboratories, Cat. 001-010-3501).

k. Skatron 96-well microtiter plate washer (Skatron, Inc.).

l. Incubator, mechanical convection (Precision Scientific Model 4EM).

m. Soxhlet extraction apparatus (Kontes 585000-023).

n. Extraction thimble, cellulose, 43 × 123 mm (Whatman 2800432).

o. Filter paper, Whatman #1, 24 cm.

NOTE: Equivalent apparatus may be substituted for those specified.

2. Instrumentation

a. Bio-Tek Model 310-96 well-plate reader with software (Bio-Tek Instruments, Inc.).

b. IBM PC/AT or compatible computer.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. Acetonitrile (ACN).

b. Hexane.

c. Benzene.

d. Dichloromethane (DCM).

e. Polyoxyethylene-sorbitan (Tween 20, Sigma Chemical Co.).

f. Sodium chloride, reagent grade.

g. Potassium phosphate, monobasic, reagent grade.

h. Potassium phosphate, dibasic, reagent grade.

i. Sodium azide (Sigma Chemical Co.).

j. Magnesium chloride hexahydrate.

k. Diethanolamine, 98% (Sigma Chemical Co.).

l. p-Nitrophenyl phosphate, 5 mg tablets (Sigma Chemical Co.). Store in a freezer at −10° C.

m. Alumina oxide (Bio-Rad acid alumina AG-4 100-200 mesh). Extract with dichloromethane for 24 hours in a soxhlet apparatus. Dry at 130° C for 24 hours; then store at 110° C.

n. Ovalbumin, crude powder, grade II (Sigma Chemical Co.).

o. Anti-mouse IgG (whole molecule) alkaline phosphatase conjugated, affinity isolated antigen, specific antibody developed in goat (Sigma Chemical Co.). Store at 4° C.

p. Py-1 antibody, 1 mg/mL. Monoclonal anti-pyrethroid antibody is referred to as Py-1 (Stanker, L. H., et al., *J. Agr. Food Chem.* 37:834-839, 1989). Py-1 is produced by the corresponding hybridoma cell line as an ascites fluid. The antibody is purified and stored at −20° C as a stock solution at a concentration of 1 mg/mL in small aliquots. Once an aliquot is thawed, it should be stored at 4° C. When used in the competition ELISA described in this method, using permethrin as a competitor, an I_{50} of 1.55 ng ± 0.6 ng should be observed. (For source of supply, contact Chemistry Division, FSIS.)

q. Coating antigen stock solution, 5 mg/mL: 3-phenoxyacetic acid conjugated to bovine serum albumin (3-pba-BSA) in PBS-7. The quality of each lot of conjugate must be evaluated in a c-ELISA using permethrin as a competitor and the monoclonal antibody Py-1. An I_{50} of 1.55 ng ± 0.6 ng should be observed. Store at −20° C in small aliquots. (For source of supply, contact Chemistry Division, FSIS.)

NOTE: All solvents should be certified for use in pesticide analysis.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

2. Solutions List

a. Tween 20, 1% solution. Dilute 1 mL Tween 20 to 100 mL with distilled water.

b. Tween 20, 0.05% wash solution. Dilute 1 mL of Tween 20 to 2 L with distilled water.

c. Phosphate buffered saline, pH 7.0 (PBS-7). Combine 9.65 g Na_2HPO_4, 1.9 g NaH_2PO_4, and 42.5 g NaCl. Add 9 L of distilled water and ajdust the pH to 7.0 using 1N HCl or NaOH. Dilute to 10 L with distilled water.

d. Assay buffer. Dilute 1 mL of 1% Tween 20 solution to 1 L with PBS-7 and mix thoroughly. Store at 4° C.

e. Phosphate buffered saline, pH 9.0 (PBS-9). Add 61.4 g NaCl and 18.76 g Na_2HPO_4 to 6 L of distilled water, and adjust the pH to 9.0 using 1N NaOH. Adjust volume to 7 L. Store at 4° C.

f. Diethanolamine buffer, 10%. Add 97 mL of diethanolamine, 0.2 g NaN_3, and 0.1 g $MgCl_2 \bullet 6H_2O$ to 800 mL distilled water. Adjust pH to 9.8 with 6N HCl and adjust final volume to 1 L. The buffer is filtered (Whatman #1) and stored at 4° C.

g. pba-BSA, 5 μg/mL solution. Dilute 100 μL of the 5 mg/mL pba-BSA stock solution to 100 mL with distilled water. This provides enough solution to coat ten 96-well plates. Prepare fresh prior to coating plates.

h. Ovalbumin, 3% in PBS-9. Add 4.5 g of ovalbumin powder to a 200-250 mL flask or bottle containing 150 mL of PBS-9 buffer at 30-40° C, cap, and mix until dissolved. Filter through glass wool and collect in a stoppered bottle. Store at 4° C.

i. Py-1 antibody, 1:5000 dilution. Dilute 3 μL Py-1 antibody (1 mg/mL) to 15 mL with assay buffer. Prepare immediately before use.

j. Antimouse IgG, 1:500 dilution in assay buffer. Dilute 30 μL of antimouse IgG, 5 mg/mL, to 15 mL with assay buffer. Prepare immediately before use.

k. p-Nitrophenyl phosphate solution, 1 mg/mL. Dissolve three 5 mg tablets of p-nitrophenyl phosphate in 15 mL 10% diethanolamine buffer. Prepare immediately before use.

l. ELISA control solution. 6% ACN in assay buffer. Dilute 6 mL acetonitrile to 100 mL with assay buffer.

m. Extraction solvent, 85% acetonitrile/water. Add 850 mL acetonitrile to a 1 L volumetric flask or mixing cylinder and dilute to volume with distilled water.

n. 2% NaCl solution. Add 20 g NaCl to a 1 L volumetric flask or mixing cylinder and dilute to volume with distilled water.

o. 6% acetonitrile/assay buffer. Add 6.0 mL of acetonitrile to a 100 mL volumetric flask and dilute to volume with assay buffer.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

3. Preparation of Microtiter Plates

a. Using a multichannel pipet, add 100 μL of a 5 μg/mL solution of pba-BSA in distilled water to each well of a 96-well microtiter plate.

b. Place the plate, uncovered, in an incubator at 37° C, and allow the pba-BSA solution to evaporate overnight. Make sure that all traces of moisture are gone before removing plate.

c. Store ''coated'' plates in a sealed container at 4° C, and use within 4 weeks.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Permethrin (mixed isomers) and other synthetic pyrethroids may be obtained from:

Director
Environmental Monitoring Support Laboratory
Office of Research and Development
Cincinnati, OH 45268

2. Preparation of Standards

a. Permethrin stock standard, 0.5 mg/mL permethrin. Accurately weight out 25 mg of the permethrin standard (mixed isomers) into a 50 mL beaker and dissolve in ca. 30 mL acetonitrile. Transfer quantitatively to a 50 mL volumetric flask and dilute to volume with acetonitrile.

b. Permethrin intermediate standard, 10 μg/mL. Pipet 1.0 mL of permethrin stock standard into a 50 mL volumetric flask and dilute to volume with acetonitrile.

c. Permethrin fortification standard, 2.5 μg/mL. Pipet 5 mL of 10 μg/mL permethrin standard into a 25 mL volumetric flask. Pipet 15 mL acetonitrile into the flask (20 mL final volume) and mix.

d. Permethrin ELISA standard, 0.3 μg/mL. Prepare a 0.30 μg/mL standard to be used in constructing a standard curve by adding 60 μL of the 10 μg/mL permethrin standard into a 15 mL centrifuge tube and diluting to 2 mL with assay buffer.

3. Storage Conditions

Store all standards at 4° C.

4. Shelf Life Stability

a. Stock, intermediate, and fortification standards are stable for at least 6 months.

b. Permethrin ELISA standard must be prepared fresh daily.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Pass chilled sample through a food chopper twice. Plug a powder funnel with glass wool. Put approximately 100 g of the comminuted fat into the funnel, place the funnel in a beaker, and render at 100-110° C overnight or until the fat ceases to drop.

2. Sample Extraction

a. Add 2.5 g of rendered fat into a 50 mL centrifuge tube. Add 25 mL 85% acetonitrile/water extraction solvent, cap securely, and place tube into a 55° C water bath for 5 min.

b. Shake sample vigorously for 3 min. A mechanical shaker may be used for this operation if it has a cycle speed greater than 200 strokes/min.

c. Immediately centrifuge sample for 2 min at 3000 rpm. Chill sample until fat has solidified. (Do not chill to point of phase separation or freezing of water phase.)

d. Remove 10 mL of the liquid phase and place in a 125 mL separatory funnel. No suspended particles of fat should be transferred.

e. Add 50 mL 2% NaCl solution and swirl. Add 15.0 mL hexane, stopper, and shake for 2 min.

f. Carefully drain and discard the aqueous fraction.

g. Add 10 mL of distilled water to the separatory funnel and shake gently for 30 sec. Allow phases to separate completely.

h. Drain and discard the 10 mL wash. A small amount of hexane may be drained to displace residual water in the stopcock.

i. Drain the hexane phase into a 15 mL graduated centrifuge tube. Adjust volume to 15 mL with hexane.

j. Transfer the hexane eluate to a 1 cm × 0.5 cm id column of alumina topped with 2 cm sodium sulfate. Avoid transferring any residual water that may be in the tip of the centrifuge tube. If less sensitivity is required, a suitable aliquot of the eluate may be diluted to 15 mL and applied to the column.

k. Wash the column with two 5 mL portions of hexane. Discard the washes.

l. Elute permethrin from the column into a 15 mL centrifuge tube using 5 mL benzene.

m. Evaporate the organic solvent to dryness under a stream of nitrogen in a 40° C water bath. Dissolve the residue in 60 μL of acetonitrile, then dilute to 1.0 mL with assay buffer.

n. Analyze sample extracts for permethrin concentration using the competition ELISA described in section F.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

3. Flow Chart Summary

Cleanup Flow Chart

- 2.5 g sample in 50 mL centrifuge tube
  - Add 25 mL ACN/water
  - Heat 5 min at 55° C
  - Extract 3 min
  - Centrifuge
  - Chill to 4° C
- Chilled sample
  - Take 10 mL aliquot
- Aliquot (10 mL)
  50 mL 2% NaCl
  15 mL hexane
  - Shake 2 min
  - Discard aqueous phase
  - Backwash 10 mL H_2O
  - Discard aqueous phase
  - Drain hexane on column
- Alumina column
  - Discard hexane eluate
  - Wash 2 × 5 mL hexane (discard washes)
  - Elute 5 mL benzene
- Benzene in 15 mL tube
  - Take to dryness
  - Dissolve in 60 µL ACN
  - Add 940 µL buffer
- Go to ELISA

ELISA Flow Chart

- Microtiter plate
  - Add 100 µL pba-BSA
  - Dry overnight at 37° C
- pba-BSA coated plate
  - Add albumin solution
  - Incubate 1 hr
  - Wash 3X
- Albumin-treated plate
  - Add standards, controls
  - Add 100 µL samples
  - Add 100 µL antibody
  - Seal plate
  - Incubate 1 hour (37° C)
  - Wash 7 ×
- Plate + residual antibody
  - Add 50 µL IgG
  - Seal plate
  - Incubate 1 hr (37° C)
  - Wash 7x
- Plate reacted with IgG
  - Add 100 µL p-nitroso-phenyl phosphate
  - Wait 30 min
  - Read absorbances with plate reader
  - Calculate results

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Competition ELISA

NOTE: All solutions used should be brought to room temperature before being added to microtiter plates.

a. Add 400 μL of 3% ovalbumin solution to each well of an antigen-coated microtiter plate. Incubate the plate at 25-37° C for 1 hour. Wash the plate 3 times with 0.05% Tween 20/water solution. Remove residual wash solution by inverting the plate and rapping repeatedly onto an absorbent surface (e.g., paper towels).

b. Refer to the plate map in section F.2 below. Add 100 μL of 6% acetonitrile in assay buffer to columns 1, 2, and 12, and to all wells in row A except A3. Add 200 μL of 0.3 μg/mL standard solution to well A3.

c. Prepare a standard curve by making seven serial 1:2 dilutions. Transfer 100 μL of standard from well A3 to well A4, and mix by repeated pipetting. Repeat the procedure for wells A4-A5, A5-A6, A6-A8 (A7 is a control), A8-A9, A9-A10, and A10-A11. Remove 100 μL from A11 and discard. (Standard in well A3 is equivalent to 300 ppb permethrin in the sample unless an aliquot is taken in section E.2, step j.

d. All samples are analyzed in triplicate. Using the plate map in F.2 as a template, add 100 μL aliquots of each sample to each of the three specified wells. After all samples have been applied, any empty wells remaining can be filled with 100 μL of 6% acetonitrile in assay buffer. These will serve as additional controls.

e. Add 100 μL of a 1/5000 dilution of the 1 mg/mL stock solution of Py-1 in assay buffer to all wells except those in column 1. Column 1 is the no primary antibody blank.

f. Seal the plate using a plate sealer or other airtight wrap and incubate for 1 hour at 37° C.

g. Wash the plate 7 times with 0.05% Tween 20 solution. Remove residual wash solution as in step a above.

h. Pipet 50 μL of alkaline phosphatase conjugated goat-antimouse immunoglobulin diluted 1/500 in assay buffer into all wells of the plate.

i. Seal and incubate the plate at 37° C for 1 hour; then wash as in step g.

j. Add 100 μL of a 1 mg/mL solution of p-nitrophenyl phosphate (freshly prepared in 10% diethanolamine buffer) to all wells. Allow color to develop at room temperature for 30 minutes, or until the absorbance of the controls is in the range 1.0-2.0; then record the absorbances of all wells in dual wavelength mode at 405 nm (540 nm reference) using a plate reader.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

2. ELISA Plate Distribution Map

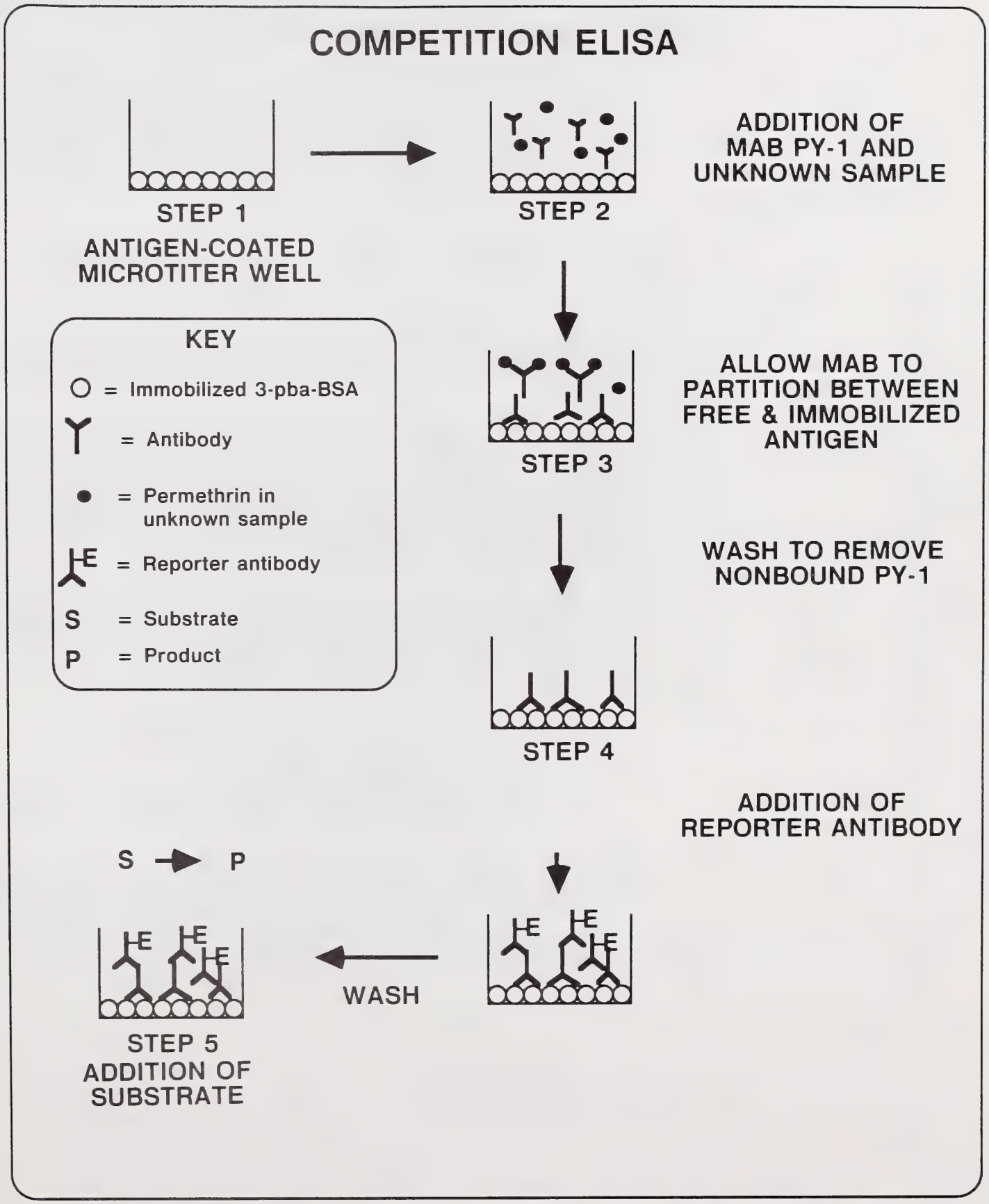
	1	2	3	4	5	6	7	8	9	10	11	12
A	B	C	S	S/2	S/4	S/8	C	S/16	S/32	S/64	S/128	C
B	B	C	1	2	3	4	5	6	7	8	9	C
C	B	C	10	11	12	13	14	15	16	17	18	C
D	B	C	19	20	21	1	2	3	4	5	6	C
E	B	C	7	8	9	10	11	12	13	14	15	C
F	B	C	16	17	18	19	20	21	1	2	3	C
G	B	C	4	5	6	7	8	9	10	11	12	C
H	B	C	13	14	15	16	17	18	19	20	21	C

B = Blanks (no antibody; 6% acetonitrile/assay buffer)
C = Controls (6% acetonitrile/assay buffer)
S = Standard (30 ng/well)
1-21 = Samples applied to plate (each sample is applied three times)

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

3. Competition ELISA Flow Chart



DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

NOTE: Since the ELISA antibody integrates its response over the entire spectrum of compounds that it recognizes, it is not possible to determine the identity or actual concentration of the pyrethroids present in a positive sample. All positives are quantitated as ppb permethrin.

a. Each row on the plate is treated as a separate experiment. Determine an average control value for each row by averaging all acceptable control values in the row. Any individual value that appears to be an outlier may be tested and discarded. Once average values have been determined, the percent inhibition for all wells in columns 3-6 and 8-11 can be calculated using the formula:

$$\text{Percent inhibition} = \frac{[\text{Abs. (control)} - \text{Abs. (sample)}]}{\text{Abs. (control)} - \text{Abs. (blank)}} \times 100\%$$

b. Average the three separate values of percent inhibition for each sample. Any individual value that appears to be an outlier may be tested and discarded.

c. Using semi-log paper, plot concentrations of the permethrin standards in ng/well (X axis) versus their percent inhibition values. Connect all points to form a standard curve. Concentrations (as permethrin) of all samples considered positive (see NOTE below) can be determined graphically by constructing a drop line from the sample's percent inhibition value on the standard curve to the X axis.

Convert concentrations to ppb permethrin by dividing the permethrin concentrations in ng/well by the sample weight in grams/well. If no dilutions are made in section E.2, step j, this value is 0.1 g. If dilutions were made, this value may be calculated using the formula:

g sample/well = 2.667(W)(A)/1000V, where

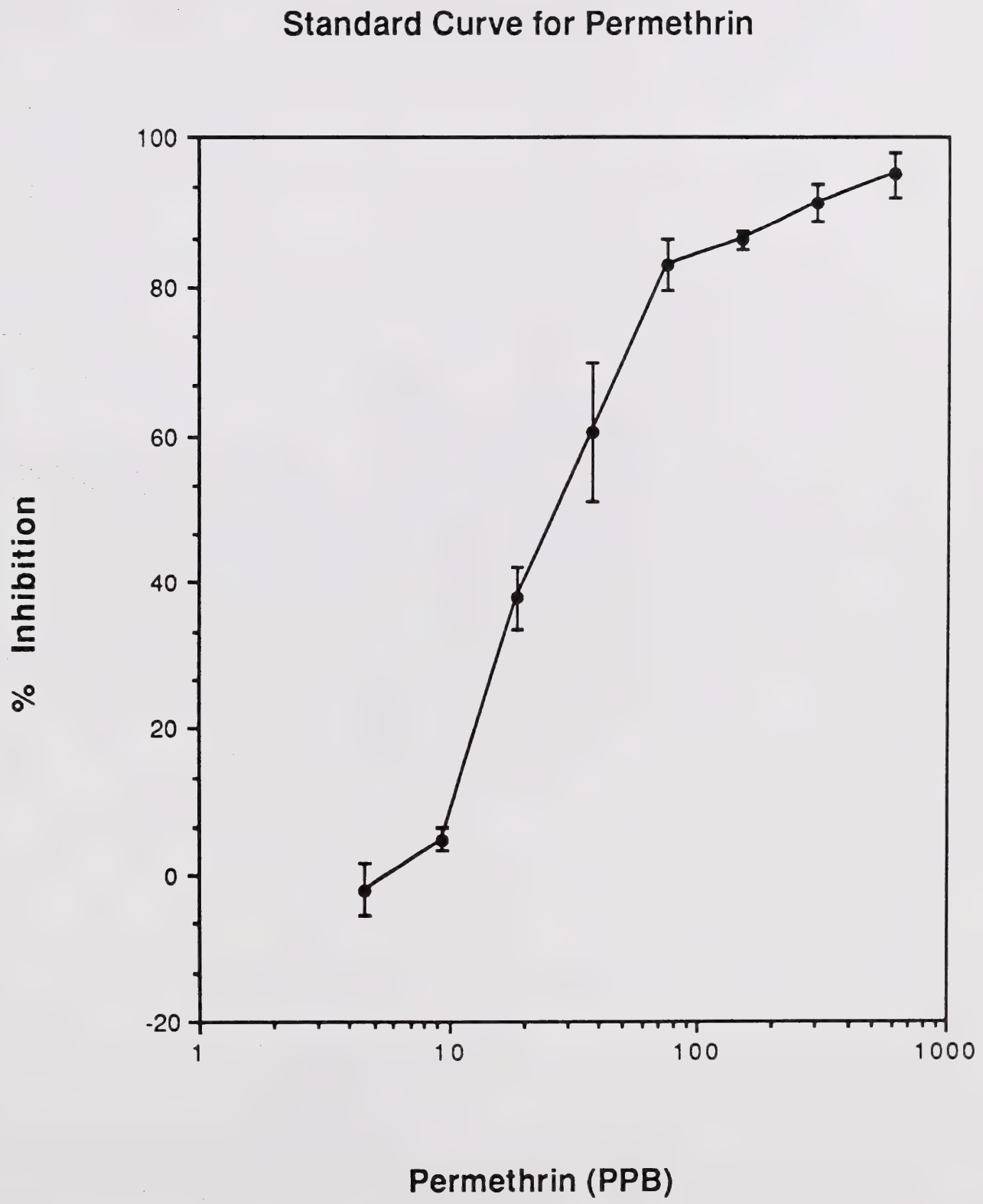
W = sample weight in grams
A = mL extract from E.2.i applied to alumina column
V = final volume of extract in mL

NOTE: It is not uncommon for blank samples to exhibit high background readings. Percent inhibition readings as high as 15-20% have been observed with some blank fat samples. Samples giving inhibition values at or below this level range are likely to show a high incidence of false positives. Reliable detection of probable positive samples requires > 25% inhibition of response.

DETERMINATIVE METHOD

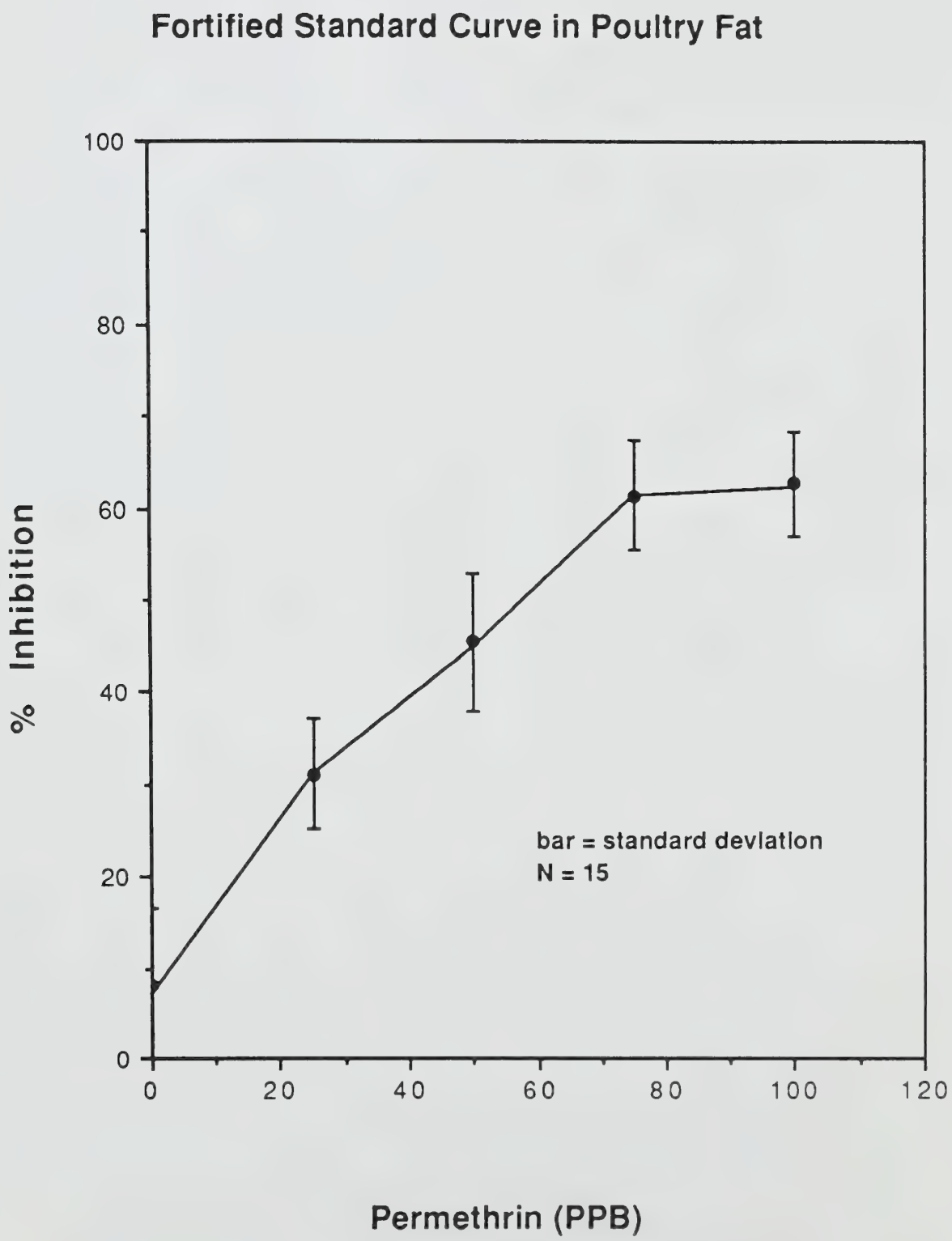
G. CALCULATIONS (Continued)

2. Standard Curves



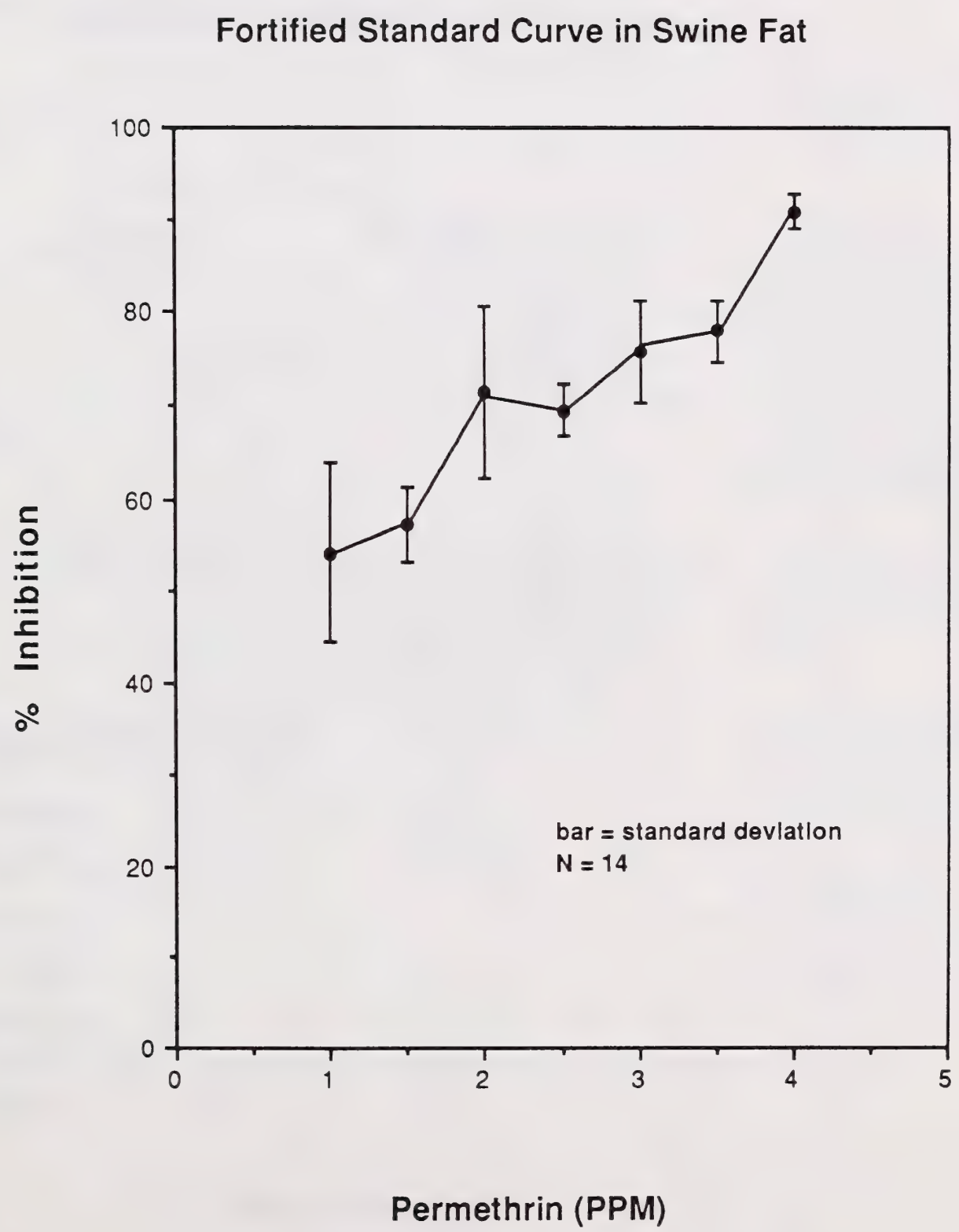
DETERMINATIVE METHOD

G. CALCULATIONS (Continued)



DETERMINATIVE METHOD

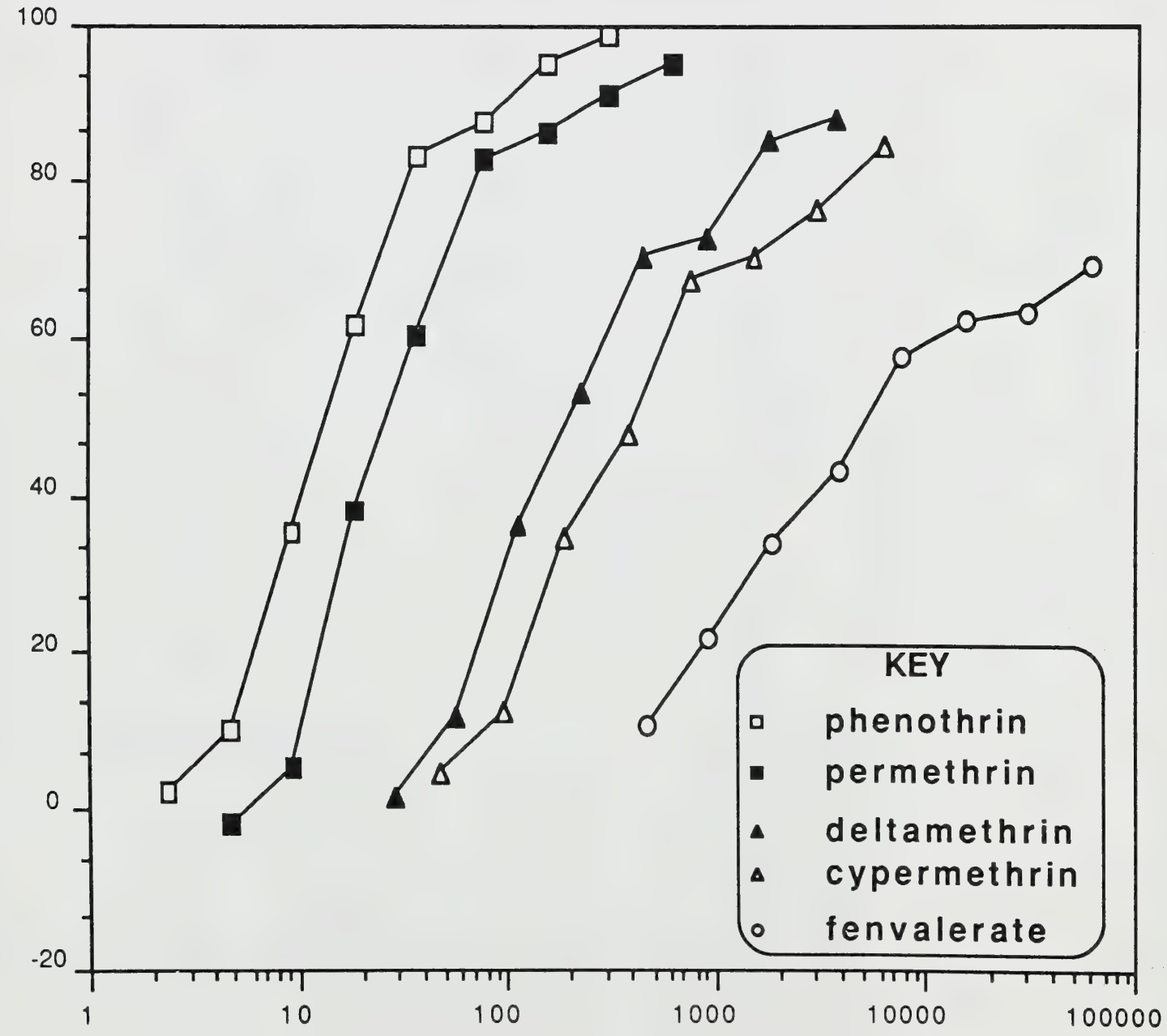
G. CALCULATIONS (Continued)



DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

Standard Curves for Synthetic Pyrethroids



DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Immunoassay Determination of Synthetic Pyrethrins by C-ELISA		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Benzene Acetonitrile Hexane	These solvents are all flammable and can produce toxic effects through exposure to the skin, eyes, respiratory system, or ingestion. Benzene is a carcinogen.	All procedures involving solvents should be carried out in an efficient fume hood and away from any source of ignition. Protective gear should be worn at all steps where the possibility of solvent spillage exists.
	D. Standards	Concentrated standards may be toxic. Exposure paths are the same as described above for reagents.	Prepare standards in a fume hood or glove box. Use protective gear.
4. Disposal Procedures	Reagent mixtures used in section E	(see above)	Hold in designated cans awaiting disposal by the contractor or in-house specialist.
	Alumina columns (used)	(see above)	Allow columns to dry in a fume hood. Dispose of packing by pouring into a plastic bag and discarding.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Acceptable Recovery (%)*	*Analytical Range (ppb)*	*Repeatability %CV*
Permethrin	50-100	50-200	< 30

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Reagents, solvents (C.1)	Must be free of interfering substances
Sample weight (E.2.a)	2.50 ± 0.05 g
Column elution rate (E.2.j-l)	Less than 1.0 mL/min for all elution steps
Incubation temperature (F.1.g,j)	37° ± 3° C
Incubation conditions (F.1.g,j)	Plates must be tightly sealed prior to incubation steps

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Analyze permethrin standards at 50, 100, and 200 ppb with three replicates for each compound.

ii. Phase II: Recoveries—Fortified samples must include at least three levels with three replicates each at 50, 100, and 200 ppb using permethrin standards. Each set should include a reagent blank and tissue blank.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation—14 samples for each species monitored, including blanks and tissues fortified in the range 50-200 ppb.

(a) Samples submitted by supervisor or LSO.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

i. No false positives.

ii. No recovery missed if present at or above 50 ppb (fortified level).

iii. Refer to section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst.

ii. Blind check samples or random duplicates chosen by the supervisor or LSO. Records to be maintained by the analyst and reviewed by the supervisor and LSO:

(a) All duplicate findings.

(b) All recovery values.

(c) Running average, standard deviation, and CV for all recoveries.

(d) CUSUM charts.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase II of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Fat.

b. Sample receipt size: Sufficient tissue for all quantitative and confirmational analyses and sample reserve portion (approximately 100 g).

c. Sample receipt condition: Chilled to frozen.

d. Sample storage:

i. Time: 3 months.

ii. Condition: Store at −4° C or below until analyzed.

6. Sample Set

Each set must contain a tissue blank, a fortified blank at 50-200 ppb (100 ppb recommended), and the samples to be analyzed.

7. Sensitivity

a. Lowest detectable limit (LDL), ppb: permethrin (mixed), 25; others, see 7.c.

b. Lowest reliable quantitation (LRQ), ppb: permethrin (mixed), 50; others, see 7.c.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

c. Py-1 antibody sensitivity for selected pyrethroids.

Compound	I_{50} *Value (ng)*	*Method LDL (ppm)*
Permethrin (mixed)	1.5	0.025
Phenothrin	1.5	0.025*
Deltamethrin	15	0.25*
Fenpropathrin	17	0.28*
Cypermethrin	30	0.50*
Flucythrinate	120	2.0*
Fenvalerate	350	5.8*
Tetramethrin	> 600	> 10*

*Estimated limits calculated using I_{50} values. Assumes similar recoveries for all compounds.

STYRENE IN MEAT BY GC/MS HEADSPACE ANALYSIS

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 4
E. Extraction Procedure 5
F. Analytical Quantitation 6
G. [Reserved]
H. Hazard Analysis 7
I. [Reserved]
J. Quality Assurance Plan 8

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Styrene can be detected at levels 1-10 ppm by gas chromatographic/mass spectrometric (GC/MS) analysis of headspace vapors from tissues exposed to styrene. The analysis is performed on a GC/MS system equipped with a 30 m OV-1 capillary column.

Styrene is soluble in organic solvents and volatizes rapidly in air. Precautions must be taken to prevent contamination of the laboratory environment and the sample during handling, preparation, and analysis.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus	a. Vials: 10 mL crimp top, Telfon sealed. b. Syringes. i. 1.0 mL (0.1 mL graduated). ii. 100 μL (10 μL graduated). iii. 10 μL (1 μL graduated). c. Forceps: Medium, sharp-pointed, 4-6″ long. d. Scalpels and blades: No. 3 industrial scalpel with No. 10-X blades. e. Cotton-tipped applicator sticks: 6″, wooden. f. Paper or cotton towels: lint-free. g. Metal pan. h. Ceramic bench top protector.
2. Equipment	a. Hewlett Packard 5992 GC/MS system, or equivalent. b. 30 m OV-1 (dimethyl silicone) capillary column, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Methylene chloride: Burdick and Jackson, or equivalent.

b. Methanol: Burdick and Jackson, or equivalent.

c. Hexane: Burdick and Jackson, or equivalent.

d. Acetone: Burdick and Jackson, or equivalent.

DETERMINATIVE METHOD

D. STANDARDS

1. Source	Styrene may be purchased from Fisher Scientific Co. or any chemical supply house. Purity must be ascertained from supplier.
2. Preparation of Control and Standard	a. Prepare a control blank by sealing an empty 10 mL crimp-top, Teflon sealed vial. b. Prepare a 10 ppm standard by sealing 0.1 μL of pure styrene in a 10 mL vial.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Preparation of Apparatus and Glassware

a. Rinse with solvent all vials, syringes, and tools that will come directly into contact with tissue samples. When rinsing syringes, use vacuum to draw solvents through syringe barrel. Use the following sequence for solvent cleaning: methylene chloride, methanol, hexane, and acetone.

b. Saturate a cotton or paper towel with solvent and wipe all surfaces with solvents as sequenced in 1.a.

c. Air-dry items with plastic or wooden parts. Dry syringes by continuously drawing a vacuum through the syringe barrel. Dry other items in an oven at 100° C for 15 min.

d. Place all cleaned items in a metal tray and allow them to cool before preparing samples.

2. Sample Preparation

a. Prepare samples by trimming 3-5 g of tissue from exterior of sample. Seal trimmings in a 10 mL vial. Interior trimmings can be taken to determine degree of saturation.

b. Fortify an extra tissue sample with 0.1 μL of styrene ($\fallingdotseq$ 20 ppm).

NOTE: It has been observed that meat tissue absorbs up to 99% of the styrene with which it is fortified.

3. Sample Extraction

a. Place sealed vials in 100° C oven for 15 min prior to injection and analysis.

b. Rinse a 100 μL syringe in the solvents as sequenced in 1.a. Remove plunger and place barrel and plunger in a 100° C oven for 30 sec. Prolonged heating of the syringe may cause the seals to deteriorate and leak.

c. Inject 100 μL of headspace from control blank 10 mL vial.

d. Repeat step b.

e. Inject 100 μL of headspace from a tissue sample.

f. Repeat step b.

g. Inject 100 μL of empty syringe to check syringe.

h. Repeat step b.

i. Repeat steps e, f, and g with 10 μL injections of samples.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions	GC parameters. a. Injection port temperature: 160° C. b. Oven temperature 1: 70° C for 1 minute. c. Oven temperature 2: 240° C, programmed at 16° C/min and hold for 1 min. d. Solvent elution time: 1.0 min. e. Vent delay: 60 sec. f. Column pressure: 15 psi. g. Retention time of styrene: 1.6 min.
2. General Operation	a. Run GC/MS system through a ''dry run'' analysis, without injecting, to assure minimal column bleed at monitored masses (m/z = 51, 52, 77, 78, 103, and 104). b. Monitor masses 51, 52, 77, 78, 103, and 104 in SIM mode with a dwell time of 200 milliseconds. c. Set EM voltage at 200 eV above autotune voltage. d. Using the above parameters, a positive finding is indicated by a peak for styrene at all monitored masses with a retention time of 3 to 4 min.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Styrene in Meat by GC/MS Headspace Analysis		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Methylene chloride Methanol Hexane Acetone	These reagents are flammable and corrosive, and the vapors are irritating to the skin, eyes, and respiratory system.	These solvents should only be used in an efficient fume hood, away from any heat-generating devices.
	D. Standards		
	Styrene	May induce nausea or narcosis.	This chemical should be handled in a fume hood.
4. Disposal Procedures	Organic solvent mixtures	(see above)	Segregate chlorinated from nonchlorinated and hold in designated storage cans until disposed of by the contractor or in-house specialist.
	Styrene	(see above)	Store separately in a prominently marked container until disposed of by the contractor.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Because this is a semiquantitative procedure, normal performance standards do not apply.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Preparation of apparatus and glassware.	Solvent-rinse in sequence with methylene chloride, methanol, hexane, and acetone.

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standard, show that instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples submitted by FSIS Science & Technology Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this test.)

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division is required to commence official analyses.

b. Acceptance criteria.

No false positives or negatives.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample per analyst per set of samples.

ii. Random replicates chosen by supervisor or Laboratory QA Officer.

iii. Records to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer: all replicate findings.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	iii. Take corrective action. iv. Repeat Phase III of section J.3 above if cause was analyst-related.
5. Sample Acceptability and Stability	a. Matrix: As required by exposure incident. b. Sample receipt size: Varied; enough to accommodate all required testing. c. Condition upon receipt: Frozen. d. Sample storage: i. Time: Maximum unknown; analyte dissipates from matrices. ii. Condition: Frozen.
6. Sensitivity	1-10 ppm.

SULFONAMIDES

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 3
C. Reagents and Solutions 5
D. Standards 6
E. Extraction Procedure 8
F. Analytical Quantitation 11
G. Calculations 15
H. Hazard Analysis 16
I. Worksheet 17
J. Quality Assurance Plan 21

II. Confirmatory Method

A. Introduction 24
B. Equipment 25
C. Reagents and Solutions 26
D. Standards 27
E. Sample Preparation 27
F. Analytical Confirmation Procedure 29
G. [Reserved]
H. Hazard Analysis 35
I. Worksheet 37
J. Quality Assurance Plan 41

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

A tissue residue assay has been developed for sulfonamides using thin layer chromatography with fluorometric scanning densitometry for quantitation. After addition of an internal standard, the tissue is extracted with ethyl acetate. The sulfonamides are then partitioned into glycine buffer. After pH adjustment, the aqueous phase is extracted with methylene chloride. Separation of the drugs from coextractives is carried out on a silica gel plate containing a preadsorbent spotting area. Visualization is accomplished using UV light after dipping in fluorescamine solution.

2. Applicability/Acronyms The following acronyms will be used throughout this method.

Sulfonamides	*Acronym*	*Species/Tissues*	*RR_f*
Sulfapyridine	SPY	All/liver, muscle	1.00
Sulfadiazine	SDZ	Red meat/liver, muscle	1.12
Sulfathiazole	STZ	Red meat/liver, muscle	0.88
Sulfamerazine	SRZ	Red meat/liver, muscle	
Sulfamethazine	SMZ	All/liver, muscle	1.27
Sulfachloropyridazine	SCP	Red meat/liver, muscle	1.24
Sulfamethoxypyridazine	SMP	Red meat/liver, muscle	1.25
Sulfaquinoxaline	SQX	Poultry/liver, muscle	1.34
Sulfadimethoxine	SDM	Red meat/liver, muscle	1.43
Sulfaethoxypyridazine	SEP	Red meat/liver, muscle	1.36
Sulfaphenazole	SPZ	Red meat/liver, muscle	1.39
Sulfatroxazole	STX	Red meat/liver, muscle	1.38
Sulfisoxazole	SFX	Red meat/liver, muscle	1.32
Sulfadoxine	SDX	Red meat/liver, muscle	1.48

NOTE: RR_f = Relative retention factor (normalized to sulfapyridine).

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

A tissue residue assay has been developed for sulfonamides using thin layer chromatography with fluorometric scanning densitometry for quantitation. After addition of an internal standard, the tissue is extracted with ethyl acetate. The sulfonamides are then partitioned into glycine buffer. After pH adjustment, the aqueous phase is extracted with methylene chloride. Separation of the drugs from coextractives is carried out on a silica gel plate containing a preadsorbent spotting area. Visualization is accomplished using UV light after dipping in fluorescamine solution.

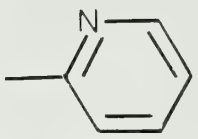
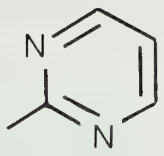
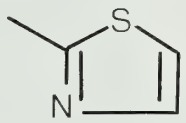
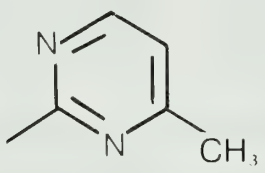
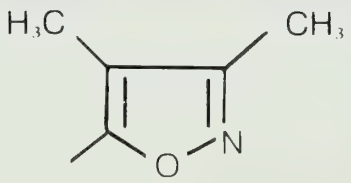
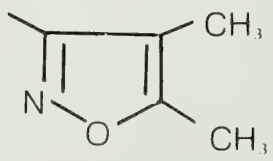
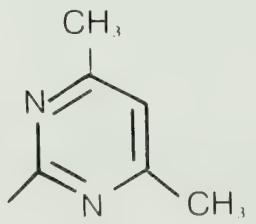
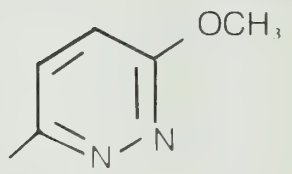
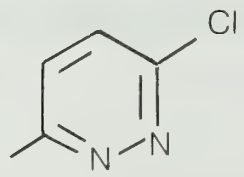
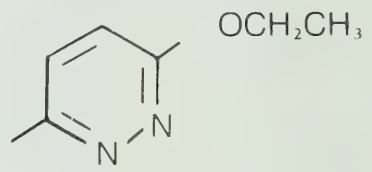
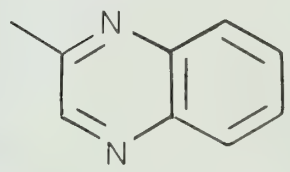
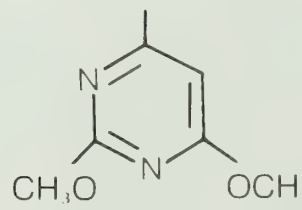
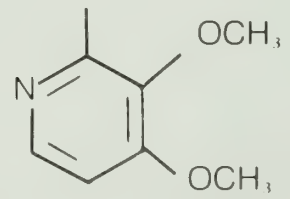
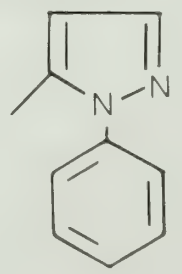
2. Applicability/Acronyms The following acronyms will be used throughout this method.

Sulfonamides	*Acronym*	*Species/Tissues*	*RR_f*
Sulfapyridine	SPY	All/liver, muscle	1.00
Sulfadiazine	SDZ	Red meat/liver, muscle	1.12
Sulfathiazole	STZ	Red meat/liver, muscle	0.88
Sulfamerazine	SRZ	Red meat/liver, muscle	
Sulfamethazine	SMZ	All/liver, muscle	1.27
Sulfachloropyridazine	SCP	Red meat/liver, muscle	1.24
Sulfamethoxypyridazine	SMP	Red meat/liver, muscle	1.25
Sulfaquinoxaline	SQX	Poultry/liver, muscle	1.34
Sulfadimethoxine	SDM	Red meat/liver, muscle	1.43
Sulfaethoxypyridazine	SEP	Red meat/liver, muscle	1.36
Sulfaphenazole	SPZ	Red meat/liver, muscle	1.39
Sulfatroxazole	STX	Red meat/liver, muscle	1.38
Sulfisoxazole	SFX	Red meat/liver, muscle	1.32
Sulfadoxine	SDX	Red meat/liver, muscle	1.48

NOTE: RR_f = Relative retention factor (normalized to sulfapyridine).

I. DETERMINATIVE METHOD

A. INTRODUCTION (Continued)

Sulfapyridine $C_{11}H_{11}N_3O_2S$ 249.3	
Sulfadiazine $C_{10}H_{10}N_4O_2S$ 250.3	
Sulfathiazole $C_9H_9N_3O_2S$ 255.3	
Sulfamerazine $C_{11}H_{12}N_4O_2S$ 264.3	
Sulfisoxazole $C_{11}H_{13}N_3O_3S$ 267.3	
Sulfatroxazole $C_{11}H_{13}N_3O_3S$ 267.3	
Sulfamethazine $C_{12}H_{14}N_4O_2S$ 278.3	
Sulfamethoxypyridazine $C_{11}H_{12}N_4O_3S$ 280.3	
Sulfachlorpyridazine $C_{10}H_9ClN_4O_2S$ 284.7	
Sulfethoxypyridazine $C_{12}H_{14}N_4O_3S$ 294.3	
Sulfaquinoxaline $C_{14}H_{12}N_4O_2S$ 300.3	
Sulfadimethoxine $C_{12}H_{14}N_4O_4S$ 310.3	
Sulfadoxine $C_{12}H_{14}N_4O_4S$ 310.3	
Sulfaphenazole $C_{15}H_{14}N_4O_2S$ 314.35	

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted for the following apparatus if necessary:

a. VIS-UV-2 chromatogram analyzer (CAMAG 76503 or Farrand).

b. IEC PR 6000 centrifuge (3000 rcf).

c. Temperature-controlled heating strip capable of 85° C, AIS multispotter.

d. Whatman LK6D silica gel plates, 20 × 20 cm. Plates have to be prewashed in methanol before use.

e. pH meter.

f. Vacuum aspirator with trap employing disposable Pasteur pipet.

g. 50 mL screw-cap polypropylene centrifuge tubes (Corning #25330, Scientific Products #C3973-50).

h. Mechanical shaker, horizontal.

i. Forced draft oven capable of 100° C.

j. TLC developing tank (2).

k. Stainless steel dipping tank.

l. Organomation N-Evap for evaporation by nitrogen and heat.

m. Vortex test tube mixer.

n. Syringes (100 to 10 μL).

o. UV light box.

p. Styrofoam ice chest or incubator set at 25-30° C for insulating TLC tank during development.

q. Chromatography paper (Whatman No. 1) or filter paper (Whatman No. 3). Use to line the developing tank to ensure adequate vapor equilibration.

r. Micropipet, Rainin P-100 adjustable.

2. Instrumentation

Although most densitometers capable of fluorescence detection can be used for the procedure, experience has shown that certain instrument configurations are superior in terms of baseline drifts, noise, etc.

I. DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

In order of decreasing desirability:

a. Double monochromator system (on both exciter and emission light path).

b. Single monochromator system (on excitation source) and interference or sharp cutoff filter (on emission side). Slit width should be adjusted to be as close as possible to full lane (8 mm on a LK6D plate). This approach minimizes errors due to sample distribution effects across the band width from spotting technique.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Ethyl acetate: HPLC or GC grade.

b. Methylene chloride: HPLC or GC grade.

c. Chloroform: distilled in glass with no ethanol preservative added.

d. Tert-butanol: reagent grade.

e. Methanol: HPLC or GC grade.

f. Acetone: HPLC or GC grade.

g. Hydrochloric acid (HCl), concentrated: reagent grade.

h. Sodium hydroxide (10N): from reagent grade chemical.

i. Fluorescamine dipping solution: 30 mg fluorescamine (Pierce Chemical Co.) in 250 mL acetone. Solution is stable for 1 month when stored at −5° C.

j. TLC solvent system: Chloroform:tert-butanol (80:20 v/v).

k. Glycine buffer (0.2M), reagent grade: Adjust with 10N NaOH to pH 12.25 ± 0.05. If laboratory temperature drops below 68° F, it may be necessary to warm this reagent to about 40° C on a steam bath just prior to use.

l. 2M phosphate buffer: Mix 2M potassium hydrogen phosphate and 2M potassium dihydrogen phosphate so that pH is 5.25.

m. 1.7M HCl/2M phosphate buffer. Prepare 1:1 v/v and adjust pH to 1.65.

n. 0.2M phosphate buffer: Weigh 45.646 g $KH_2PO_4 \bullet 3H_2O$ and dissolve in 1000 mL distilled water (solution 1). Weigh 27.218 g KH_2PO_4 and dissolve in 1000 mL distilled water (solution 2). Adjust solution 1 with solution 2 to pH 7.55 ± 0.05. Approximately 80 mL solution 1 plus 20 mL solution 2 will result in 100 mL of pH 7.6 solution.

o. Hexane: HPLC or GC grade.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Sulfonamide standards can be purchased from:

a. Sigma Chemical Co. (SPY, SDZ, STZ, SMZ, SCP, SMP, SDM, SPZ, SFX, SRZ).

b. U. S. Pharmacopeia (SDX).

c. Pfaltz & Bauer (STX).

d. Cyanamid (SEP).

e. Merck (SQX).

2. Preparation of Standards

a. Stock solution, 1 mg/mL.

All sulfonamide standards, including the internal standard (sulfapyridine), are prepared as follows: Weigh 100 $\pm$ 0.1 mg of each sulfonamide into separate 100 mL volumetric flasks. Dissolve and bring to volume with acetone.

b. Working standards (used for fortification).

All working standards are diluted with phosphate buffer (refer to section C, Reagent and Solution List, item n).

i. 10 μg/mL.

(a) Sulfonamide mixed standard solution.

Pipet 1 mL of stock solution of each sulfonamide of interest into a 100 mL volumetric flask. Do not add internal standard. Bring to volume.

(b) Internal standard solution.

Pipet 1 mL of the internal standard (IS) solution into a 100 mL volumetric flask. Bring to volume.

ii. Solution D, 2.50 μg/mL IS.

Pipet 25 mL of the 10 μg/mL IS solution into a 100 mL volumetric flask and bring to volume.

iii. Solution C, 5.00 μg/mL sulfonamide of interest.

Pipet 50 mL of the 10 μg/mL standard of the sulfonamide or mixed sulfonamides of interest into a 100 mL volumetric flask and bring to volume.

I. DETERMINATIVE METHOD

D. STANDARDS (Continued)

iv. Solution B, 2.50 μg/mL sulfonamide of interest.

Pipet 25 mL of solution C into a 50 mL volumetric flask and bring to volume.

v. Solution A, 1.25 μg/mL sulfonamide of interest.

Pipet 25 mL of solution B into a 50 mL volumetric flask and bring to volume.

3. Storage Conditions

All standards can be stored in polyethylene bottles and refrigerated at 10° C, except for stock solution (−10° C).

4. Shelf Life Stability

a. STZ, SPY, SMZ, SDM, SQX.

i. Stock solution: 6 months.

ii. Working solutions: 1 month.

Storage stability is based upon the storage conditions mentioned in D.3.

b. All other sulfonamides.

Make fresh solutions each time they are analyzed since their stability is not known at this time.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Weigh 2.5 ± 0.1 g of frozen preground tissue into a 50 mL polypropylene centrifuge tube. Allow to thaw.

b. Fortification standard curve:

Weigh four 2.5 ± 0.1 g of blank tissue as above and use as follows:

i. Blank tissue (1).

ii. Fortified tissues (3). Fortify them as follows:

Volume Added (μL)	*Solution Used*	*μg Added*	*ppm**
100	A	0.125	0.05
100	B	0.250	0.10
100	C	0.500	0.20

*ppm is calculated using 2.50 g as sample weight.

iii. Add 100 μL of standard solution D (2.50 μg/mL) to blank and fortified tissues. This will yield 0.10 ppm IS.

NOTE: High-level samples are not concentrated during the procedure as are low-level samples. It is possible, therefore, to evaluate both with this fortification curve.

c. Samples:

i. For low level:

Add 100 μL of standard solution D (2.50 μg/mL) to all low-level samples. This will yield 0.10 ppm of IS.

ii. For high level (> 1 ppm):

Add 25 μL of the internal standard stock solution (1 mg/mL) to all high-level samples. This will yield 10 ppm of IS.

d. Allow samples to stand for 15 minutes.

e. Add 25 ± 1.0 mL ethyl acetate via repipet.

f. Seal tubes with screw cap. Shake once or twice by hand to check for leakage.

g. Shake on horizontal shaker for 20 minutes at approximately 250 cycles/minute.

h. Centrifuge 5 minutes at 2500 rpm.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

i. Decant supernatant into a clean 50 mL polypropylene centrifuge tube and add 10 mL of 0.20M glycine buffer. Discard tissue residue and original tube.

j. Cap tube and shake on mechanical shaker for 5 minutes.

k. Centrifuge for 5 minutes at 2500 rpm.

l. Remove organic phase (upper layer) with Pasteur pipet attached to vacuum aspirator. Take care to remove any solid or emulsified material remaining at interface or clinging to tube wall.

m. Add 2 mL of 1.7M HCl/2M phosphate buffer. Adjust, if necessary, to pH 5.25 $\pm$ 0.10 with buffer or 1.0N NaOH or 1.0N HCl.

n. Add 10 mL hexane. Cap and shake on mechanical shaker for 5 minutes.

o. Centrifuge for 5 minutes at 2500 rpm.

p. Remove organic phase (upper layer) with Pasteur pipet attached to vacuum aspirator. Use gentle vacuum to aspirate upper layer, especially if solids are present at the layer interface.

q. Add 10 mL methylene chloride. Cap and shake on mechanical shaker for 5 minutes.

r. If no emulsions are present, centrifuge 5 minutes at 2500 rpm. If emulsions are present, centrifuge 10 minutes at 3500 rpm.

s. Remove aqueous phase (upper layer) with Pasteur pipet attached to vacuum aspirator. Entire layer can be completely removed by tilting tube and keeping pipet aspirator at tube wall. Take care to remove any fat or particulates at the interface. *If a gelatinous plug is present between the aqueous and organic phases, do not remove or aspirate it. Poor recovery will result.*

NOTE: For sulfa levels fortified according to c.ii., skip steps t-w and proceed to section F.

t. Evaporate remaining organic phase under a stream of nitrogen on an N-Evap (40° C). When the level has declined to 5 mL, rinse the sides of the tubes with an aliquot (ca 2 mL) of methylene chloride. Repeat rinse when volume declines to approximately 2.5 mL and again at approximately 1.0-1.5 mL.

u. Evaporate just to dryness. Do not allow residue to dry out.

v. Dissolve residue in 100 μL methanol and vortex samples for 30 seconds. Allow to stand for 5 minutes so that insoluble oils settle to the bottom of the tube.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

w. Keep tubes tightly stoppered in case an additional analysis is required.

NOTE: Stopping points—within day. Any step may be used as a stopping point during the course of the day with two exceptions. It is not desirable to allow the sulfonamides to remain in the strongly basic glycine buffer for more than one hour. Likewise, the concentration to dryness step should not be allowed to go unattended. Significant losses may occur if the residue is allowed to evaporate beyond the ''just to dryness'' stage and remain that way for any length of time while heated.

2. Method Outline

2.5 g sample + IS + 25 mL EtOAc	Shake 20 min. Centrifuge 5 min. Transfer supernatant to clean 50 mL tube. Discard tissue.
Supernatant + 10 mL 0.2M glycine buffer	Shake 5 min. Centrifuge 5 min. Aspirate and discard organic phase.
Glycine extract + 2 mL 1.7M HCl/ 2M phosphate buffer	Adjust pH to 5.25.
Aqueous extract + 10 mL hexane	Shake 5 min. Centrifuge 5 min. Aspirate and discard organic phase.
Aqueous extract + 10 mL CH_2Cl_2	Shake 5 min. Centrifuge 5 min. Aspirate and discard organic phase. Evaporate CH_2Cl_2 just to dryness. (Note variation for high-level samples.)
Dried residue + 100 μL MeOH	Vortex.
Spot 20 μL for TLC analysis.	

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Thin Layer Chromatography

a. Using a micropipet, spot 20 μL of each fortified control sample, the tissue blank, and up to 13 incurred tissue samples on the preadsorbent layer of the LK6D plate with the preadsorbent zone heated to 85° C on a heat strip.

Take care not to spot any of the insoluble oil fraction as this will distort the chromatography. A clean, oil-free 20 μL aliquot can be removed by tilting the tube so that the less viscous methanol separates from the oily residue.

Position the spot approximately 1.0-1.5 cm from the preadsorbent/silica gel interface. Do not spot on the outermost lanes of the plate as edge effects can distort both band shape and R_f, leading to inaccurate assessment.

NOTE: The spot must be applied in one even application such that the expanding spot just reaches the lane edges at the end of the application.

b. Develop the plate to 1 cm from the interface in methanol. Remove and dry the plate for 1 minute in an oven at 100° C.

c. Develop the plate to 6 cm from the interface in 80:20 chloroform:tert-butanol in a vapor-saturated tank contained in an insulated ice chest or incubator at 25-30° C. Dry plate for 1 minute in an oven at 100° C. Redevelop plate in chloroform:tert-butanol to 12 cm. Development must be carried out at 25-30° C to ensure consistent R_f values across the plate.

NOTE: Sulfathiazole can be distinguished from sulfapyridine using the chloroform:tert-butanol system, but unless conditions are optimal, densitometric resolution is incomplete. For this reason, the chloroform: tert-butanol solvent system (80:20) should be washed with water prior to use. The increase in polarity from the trace water absorbed by the organic phase will substantially increase the separation between sulfathiazole, sulfapyridine, and sulfamethazine. However, the resolution between sulfamethazine, sulfaquinoxaline, and sulfadimethoxine will decrease.

d. Dry plate in an oven for 1 minute at 100° C and dip the plate in the fluorescamine solution. Keep plate in the solution only 1-2 seconds after fully submerged; otherwise background color will be excessive.

NOTE: Do not dip the developed plate in fluorescamine unless it will be scanned the same day. Unacceptable diminution of fluorescence will occur overnight at room temperature on the dipped plates. This can be overcome by storing the derivatized plate overnight in a sealed plastic bag at −10° C. Quantitation from plates stored in this manner is acceptable. One can successfully spot one day and develop the next, or develop one day and dip the plate the next day.

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

e. Allow plate to develop in the dark at room temperature for 15-30 minutes. View the plate under UV light to check for recoveries and very high sulfa levels.

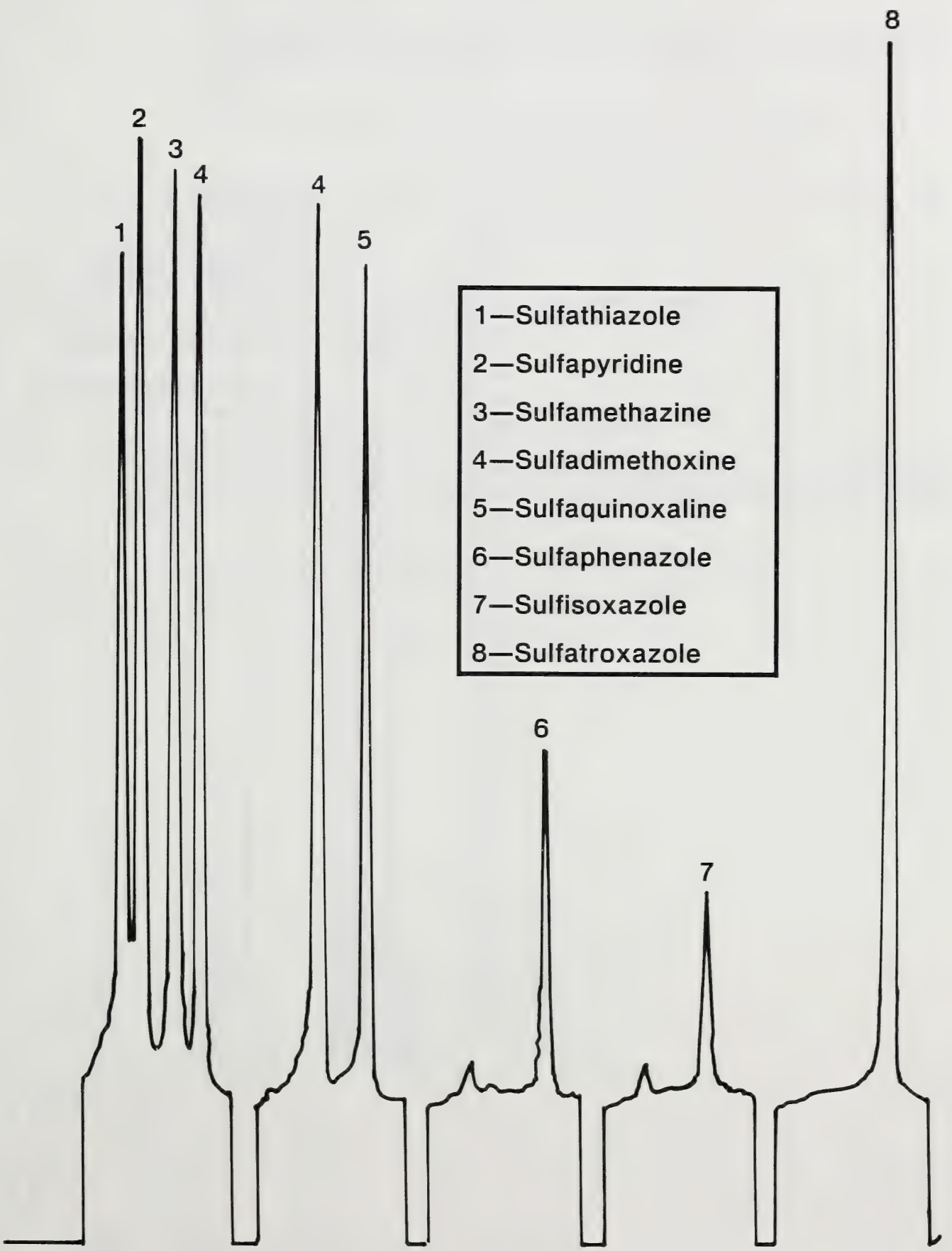
f. Scan on densitometer, using 410 nm for excitation and emission wavelengths.

g. Scan an unused lane to determine plate background for baseline correction.

I. DETERMINATIVE METHOD

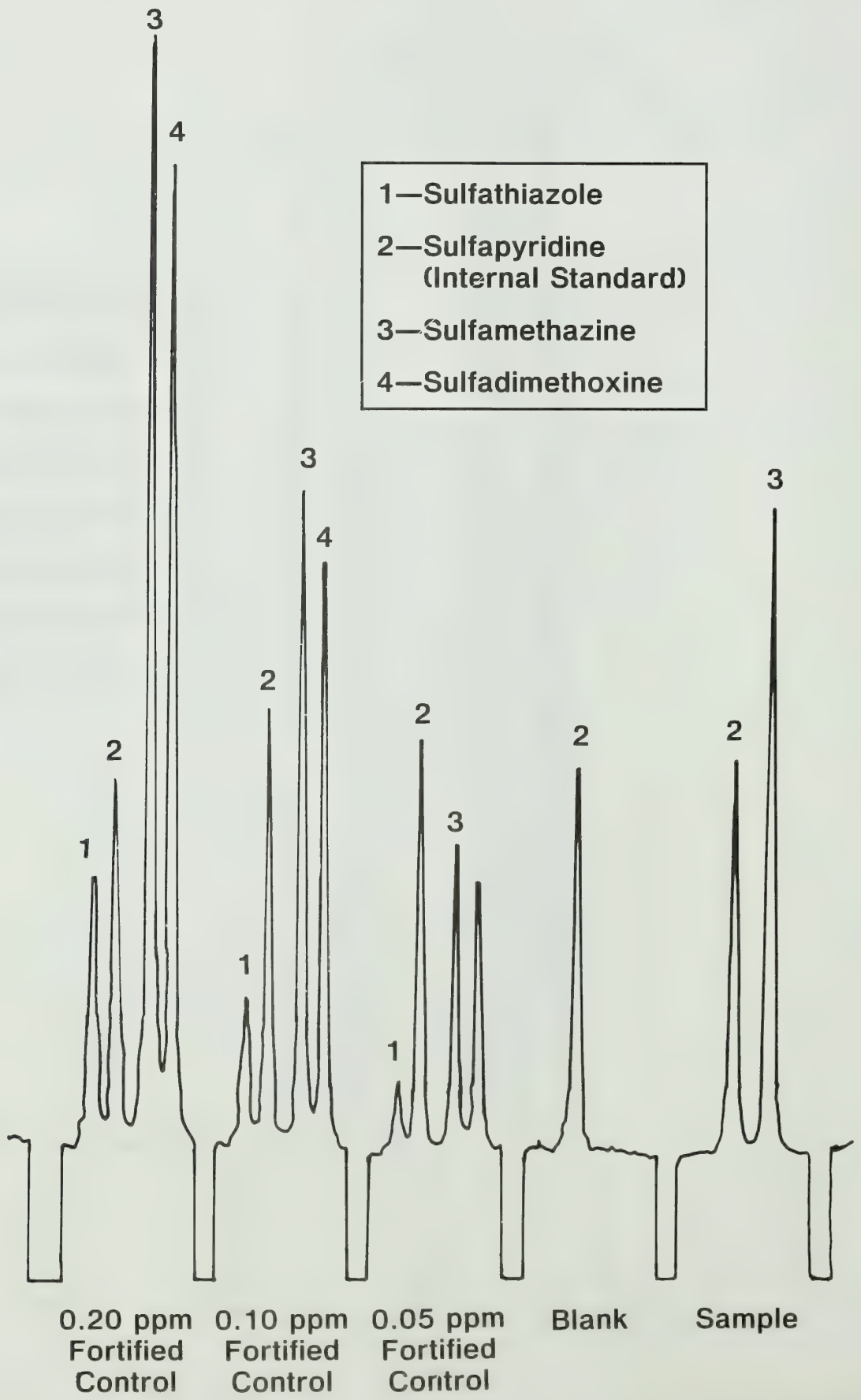
F. ANALYTICAL QUANTITATION (Continued)

2. TLC Scan of Sulfonamide Standards



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. For each plate developed, construct a standard curve from the fortified samples applied to that plate as follows:

   i. Measure the peak heights of the sulfonamides of interest and internal standard peaks of each fortified sample and calculate the peak height ratios.

   $$\text{peak height ratio} = \frac{\text{sulfonamide of interest peak height}}{\text{internal standard peak height}}$$

   ii. Using linear regression, construct a standard curve of sulfonamide concentration vs. peak height ratio.

   The equation is $y = mx + b$.
   where y = sulfonamide/IS peak height ratio
   x = sulfonamide concentration (ppm)
   m = slope
   b = y intercept

   The correlation coefficient should be ≥ 0.995.

b. Using the regression slope and intercept, compute the sulfonamide concentration (x) for each incurred sample from the measured peak height ratio.

c. For high level samples, multiply the above result by 100.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

TLC-Densitometric Procedure for Sulfonamide Residues in Animal Tissues.

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Ethyl acetate Methylene chloride Chloroform Tert-butanol Methanol Acetone	The organic reagents are very flammable and corrosive and the vapors are extremely irritating to the skin, eyes, and respiratory system.	These solvents should only be used in an efficient fume hood, away from any heat-generating devices.
Concentrated HCl Sodium hydroxide (10N)	Fumes are corrosive. Spattering may result in serious eye, skin, or respiratory damage.	pH adjustments can performed easily in a fume hood.
F. Analytical Quantitation		
Thin-layer chromatography	The hazards associated with the above solvents are increased since development cannot be done in a hood or other drafty environment.	The development tank can be set beside a hood so that plates can be removed and dried in the hood with minimum exposure to the analyst. A face mask may also be used.

4. Disposal Procedures

Organic solvent mixture	See above	Segregate chlorinated from nonchlorinated solvents as much as possible. Hold in designated containers until disposed of by the contractor or the in-house specialist.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the following page, *Sulfonamides*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

WORKSHEET: SULFONAMIDES

Analyst: _____ **Date Started:** _____ **Date Completed:** _____

Critical Control Points

Sample weight 2.5 g; 100 μL SPY Std D added; pH 5.25 unless otherwise indicated.
Room temperature: _____

Standard Curve Data

Regression analysis for S_____

b = _____

m = _____

r = _____

Regression analysis for S_____

b = _____

m = _____

r = _____

	Sample Identification	S___ Peak Height	SPY Peak Height	S___ SPY Ratio	ppm S___	S___ Peak Height	SPY Peak Height	S___ SPY Ratio	ppm S___	Comments	
1	Control .05										1
2	Control .10										2
3	Control .20										3
4	Blank										4
5											5
6											6
7											7
8											8
9											9
10											10
11											11
12											12
13											13
14											14
15											15
16											16
17											17

REMARKS:

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Element	*Analytical Range (ppm)**	*Acceptable Recovery (%)*	*Repeatability %CV*	*Reproducibility %CV*
All sulfonamides	0.02-0.2[1] 5-100[2]	5-50[3]	12	15

[1]Normal protocol.
[2]High level protocol.
[3]Varies with analyte.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Room temperature	> 68° F (If not, warm up the glycine buffer solution.)
b.	pH	5.25 ± 0.10
c.	Chamber temperature (TLC)	25-30° C

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards, minimum criteria. TLC, densitometric standard curve preparation: Four sulfonamides (sulfamethazine, sulfadimethoxine, sulfaquinoxaline, sulfathiazole) at four levels: 0, 0.05, 0.1, and 0.2 ppm.

The number of replicates is at the discretion of the supervisor.

ii. Phase II: Sample fortification (self-provided tissues), minimum criteria. Four levels—0.05 to 0.20 ppm—plus blanks.

(a) Turkey—liver and muscle—sulfaquinoxaline, sulfamethazine, and sulfadimethoxine.

(b) Swine—liver and muscle—sulfathiazole, sulfadimethoxine, and sulfamethazine.

(c) Beef—liver and muscle—sulfadimethoxine, sulfathiazole, and sulfamethazine.

The number of replicates is at the discretion of the supervisor.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Analyze 28 samples furnished by Chemistry Division.

(b) Report analytical findings to Chemistry Division, QSB.

Letter from Chemistry Division is required to commence official analysis.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

b. Acceptability criteria.

See section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents:

i. Frequency: minimum of 1 per week per analyst.

ii. Blind samples (requires dummy forms).

iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory Staff Officer.

(a) All replicate findings.

(b) Running average difference between replicates and by level present.

b. Acceptability criteria.

See section J.1 above.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Interlaboratory Check Sample Program

a. Laboratory responsibility.

i. Frequency: 1 every other month.

ii. Samples: Procured, prepared, and submitted by FSIS Science & Technology Western Laboratory (may be incurred or fortified).

iii. Report analytical findings to Chemistry Division.

b. Chemistry Division responsibility.

i. Evaluates all reported values for average, standard deviation, and coefficient of variation.

ii. Submits written reports to participating laboratories and to necessary Program officials after all reports on each set are received.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

iii. Prepares ongoing control chart by laboratory code.

iv. Submits written reports to participating laboratories and to Program officials on above items each quarter.

v. Phones and writes each laboratory submitting unacceptable values (cc: Director, Field Service Laboratory Division), indicating reason for unacceptability and requesting memo of acknow-ledgement and corrective action taken.

c. Acceptability criteria.

i. Each value per sample:

(a) Outlier based on Dixon's or Grubb's rules at the 5% significance level.

(b) Each sample's coefficient of variation at $\leq$ 12%.

ii. Acceptability of analyst—not more than 2 outliers in any 3 consecutive months.

iii. Acceptability of laboratory control—no significant statistical bias.

6. Sample Acceptability and Stability	a. Matrices: Liver, kidney, muscle, processed products. b. Sample receipt size, minimum: 10 g. c. Condition upon receipt: Cold (4.0° C). d. Sample storage: i. Time: Not more than 90 days (3 months). ii. Condition: Frozen ($<$ −5° C).
7. Sample Set	Each sample set must contain a tissue blank, a standard curve of fortified tissues at 0.05, 0.10, and 0.20 ppm, and up to 13 samples of the same species/tissue.
8. Sensitivity	a. Lowest detectable level (LDL): 0.02-0.05 ppm (varies with analyte). b. Lowest reliable quantitation (LRQ): 0.05 ppm. c. Minimum proficiency level (MPL): 0.05 ppm.

II. CONFIRMATORY METHOD

A. INTRODUCTION

1. Theory and Structures	Sulfonamides are extracted from tissue using the procedure described in Part I. The sulfonamides are first methylated with diazomethane and then acylated with pentafluoropropionic anhydride before detection and confirmation by GC/MS-EI selected ion monitoring. Generally, the three ions selected for monitoring are the 238 ion ($C_2F_5CONHC_6H_4$), which is common to all, and the M-64 and M-65 ions, which are formed when the derivatized sulfonamide loses sulfur dioxide.
2. Applicability	Refer to Determinative Method, section A.2.

II. CONFIRMATORY METHOD

B. EQUIPMENT

1. Apparatus

a. 5 mL disposable borosilicate glass centrifuge tube, Kimble #73785-5. Cap, Kimble #73802-13415.

b. N-Evap, Model 111, Organomation.

c. Diazomethane generator, Aldrich, Z10, 025-0. Distillation glassware, without ground glass joints, used to generate diazomethane from Diazald. Diazomethane is codistilled with ether.

d. Vortex test tube mixer, Lab Line Instruments, Super Mixer, Model #1290.

e. Nitrogen, high purity.

f. Syringes, 10 μL Hamilton gas-tight #1701.

g. Repipetters, Eppendorf, 100 μL, 50 μL, and 25 μL.

h. Centrifuge, Damon IEC HN-II centrifuge.

i. Disposable pipets, 5 3/4 inches, borosilicate glass, Kimble #72050.

NOTE: An equivalent may be substituted for any apparatus listed above.

2. Instrumentation

Hewlett Packard gas chromatograph 5890 with a Hewlett Packard mass selective detector 5970—electron impact (70 ev).

II. CONFIRMATORY METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane, Optima H303-4.

b. Ethyl acetate, Fisher Optima E196-4.

c. Cyclohexane, Fisher pesticide grade, C-553.

d. Diethylamine (DEA), reagent grade, Fisher, D-46.

e. 1M (pH 6.8) phosphate buffer. Prepare by making a 1M solution of Na_2HPO_4 and a 1M solution of KH_2PO_4 and mixing 1:1 (v/v).

f. Pentafluoropropionic anhydride (PFPA): Pierce #65193.

g. Diazomethane: Prepare using the generator manufacturer's instructions and the following reagents:

NOTE: Diazomethane preparation must be carried out in a working fume hood behind a protective screen or shield. Operator should wear gloves to prevent skin contact with reagents or diazomethane. Caution must be observed when handling diazomethane as it is toxic and, under some conditions, explosive. Avoid using anything with sharp edges and ground glass in any generator being used. Freshly made diazomethane solution is golden yellow in color and can be used for a maximum of 1 week. Store in freezer.

i. N-methyl-N-nitroso-p-toluenesulfonamide (PTS): reagent grade. Handle with caution. Diazald, 99%, Aldrich D2800-0.

ii. 2-(2 Ethoxy ethoxy)-ethanol (Carbitol): reagent grade, Matheson Coleman & Bell EX 210 P5755.

iii. Potassium hydroxide (KOH) 60% (W/V) aqueous, reagent grade.

iv. Ethyl ether: anhydrous reagent grade, peroxide free, Baxter, Burdick & Jackson, 106-4.

NOTE: Ethyl ether must be anhydrous. Traces of water cause decolorization of diazomethane solution with accompanying poor derivatization. Also, since diethyl ether may contain peroxides which can detonate when distilled or concentrated, the ether should be tested before using by shaking 1 mL of a freshly prepared saturated solution of potassium iodide with 9 mL of ether in a 25 mL glass-stoppered cylinder. Any yellow color indicates a concentration of peroxide greater than 0.005%, which is considered dangerous. Refer to *CRC Handbook of Laboratory Safety*, 2nd Edition, pp. 250-254, for additional information on this subject.

NOTE: An equivalent may be substituted for any reagent listed above.

II. CONFIRMATORY METHOD

D. STANDARDS

See Determinative Method, section D.

E. SAMPLE PREPARATION

Sample Preparation for Mass Spectrometric Confirmation

Using the extract prepared as outlined in the Determinative Method, section E, perform the following additional steps:

NOTE: In certain cases it may be necessary to extract a larger sample (5.0 g), or eliminate or reduce the amount of the SPY internal standard, or both. Such cases include problems of co-elution, poor recovery, or poor response. One such case is STZ, which co-elutes with SPY. (In this case, use SDM as the internal standard instead.) STZ and several other sulfonamides have characteristic low recoveries. (For these sulfonamides with either a low recovery or low response, it helps to have a clean source. The best results are obtained when the relative abundance of mass/charge of 502 of PFTBA in the autotune is between 5-10%.)

a. Transfer the remaining methanol extract from the STLC-F analysis (approximately 100 μL from the original 120 μL) to a disposable 5 mL glass centrifuge tube.

b. Rinse the 50 mL centrifuge tube twice with 0.5 mL portions of methanol and add the rinses to the extract in the 5 mL disposable glass centrifuge tube.

c. Evaporate the sample extract plus the two rinses to dryness under nitrogen.

d. Add 300 μL ethyl acetate and 1.0 mL diazomethane to the 5 mL disposable glass centrifuge. Mix. Let stand alone for 10 minutes to methylate the sulfonamides.

   NOTE: The diazomethane solution has to have been made within the last week. Its color should be a deep golden yellow—do not use if the color is a pale yellow. The diazomethane solution added to the sample should remain yellow to ensure an excess of diazomethane.

e. Reduce volume of the ethyl acetate and diazomethane solution to 300 μL with nitrogen at room temperature.

f. Add 50 μL 10% PFPA in hexane. Mix. Add 25 μL 5% diethylamine in ethyl acetate. Let stand 30 minutes to acylate the sulfonamides.

g. Evaporate to near dryness under nitrogen at room temperature. Loss of the volatile derivatized sulfonamides is risked if it is evaporated to dryness. Add 100 μL 10% ethyl acetate in cyclohexane. Mix.

h. Add 300 μL of a 1M (pH 6.8) phosphate buffer. Mix by vortexing for 15 seconds. Centrifuge at 200 G for 1 minute and quickly remove the aqueous bottom layer; otherwise, the acylation reaction will reverse at the aqueous interface. This step serves to remove the excess acylation reagents and the acylation byproduct pentafluoropropionic acid.

II. CONFIRMATORY METHOD

E. SAMPLE PREPARATION (Continued)

i. Add approximately 50 mg sodium sulfate to remove any remaining water. Mix. Centrifuge at 200 g for 1 minute.

j. Inject 2-10 μL from the organic layer for GC/MS analysis.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

a. GC parameters. (Example only. Analyst should optimize these parameters for the instrument being used.)

i. Initial column temperature: 150° C.

ii. Time at temperature 1: 0 minutes.

iii. Injector temperature: 260° C.

iv. Final temperature: 300° C.

v. Program rate: 30° C/min.

vi. Time at temperature 2: 10 minutes.

vii. Total run time: 15 minutes.

viii. Helium linear velocity: 30 cm/sec.

ix. Column.

(a) HP 19091A-102, or equivalent.

(b) Fused silica capillary column.

(c) Film: Crosslinked methyl silicone (OV-1).

(d) Film thickness: 0.33 micron.

(e) Phase ratio: 150.

(f) Column length: 25 m.

(g) Column i.d.: 0.20 mm.

b. MSD parameters.

i. Tune the MSD prior to analyzing.

ii. Examples of the MSD parameters currently being used. Analyst should optimize these parameters for the instrument being used.

(a) Transfer line: 300° C.

(b) Electron multiplier: 600 added to the autotune value.

(c) Mode: Electron impact (70 ev). Selected ion monitoring.

(d) SIM dwell time: 100 msec.

II. CONFIRMATORY METHOD

F. ANALTYICAL CONFIRMATION PROCEDURE (Continued)

(e) Program.

Time (min)	*Event*	*Sulfa monitored*
5.00	Mass spec. on.	
	Monitor ions:	
6.00-6.34	238 350 351 344 345	Sulfathiazole Sulfapyridine
6.35-6.89	238 373 374	Sulfamethazine
6.90-8.19	238 405 406	Sulfadoxine and sulfadimethoxine
8.20-15.00	238 395 396	Sulfaquinoxaline
15.00	Stop run.	

c. Inject 2-10 μL of the previously prepared external standard and plot the total ion chromatogram.

d. Inject 2-10 μL of the tissue blank and fortified tissue and plot the TIC.

e. Inject 2-10 μL of each sample and plot the TIC.

f. Determine the retention time and the peak heights of the selected ions for the sulfonamide(s) being confirmed.

2. Confirmation Criteria

a. Compare the retention time of the sample with the retention time of the fortified tissue. The times should match $\pm$ 5%. Compare the R_f of the sample with the R_f of the fortified tissue on the TLC plate. The R_f of the sample must be indistinguishable from the R_f of the fortified tissue as recorded by the densitometer.

b. The characteristic 238, M-65, and M-64 ions must be present.

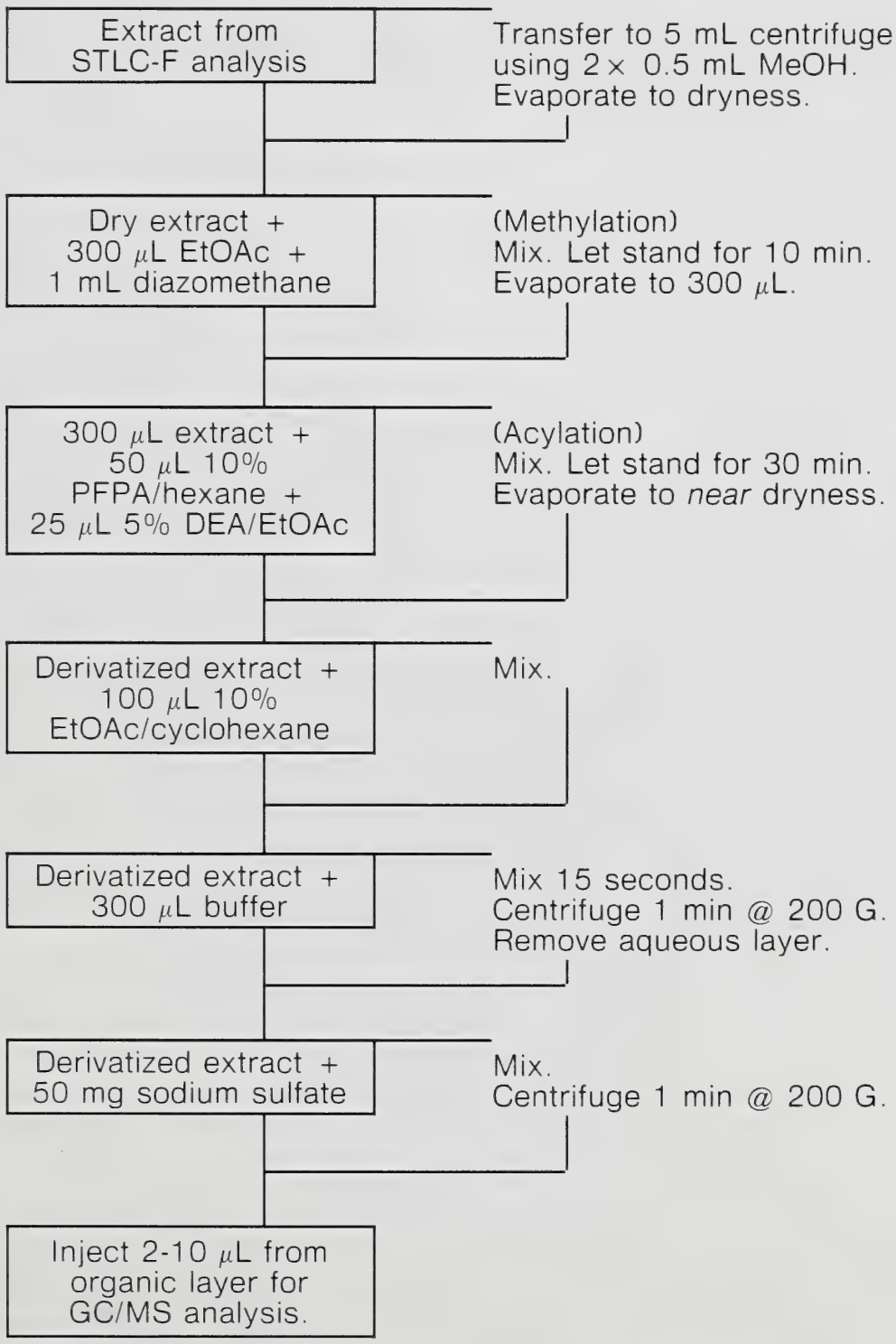
c. Compute the M-65/M-64 ion ratio for the standard, the fortified tissue, and the sample. For successful confirmation, the M-65/M-64 ratio computed for the sample must agree within $\pm$ 20% of the ratio for fortified tissue. A list of the approximate M-65/M-64 ratio expected for each sulfa is listed below.

Sulfa	*M-65/M-64 (ion ratio)*
Sulfathiazole	1.0
Sulfapyridine	5.3
Sulfamethazine	4.2
Sulfadoxine	2.5
Sulfadimethoxine	3.4
Sulfaquinoxaline	4.3

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION METHOD (Continued)

3. Method Outline



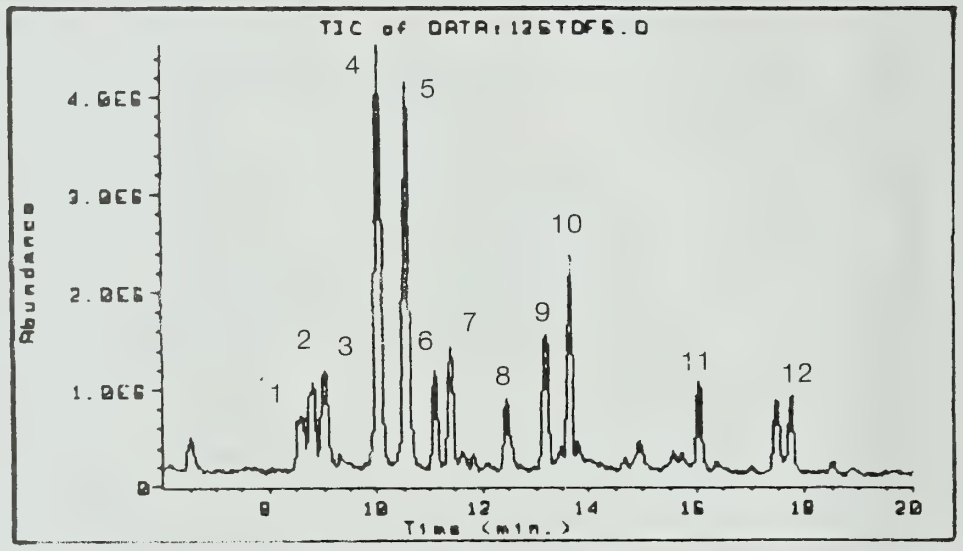
II. CONFIRMATORY METHOD

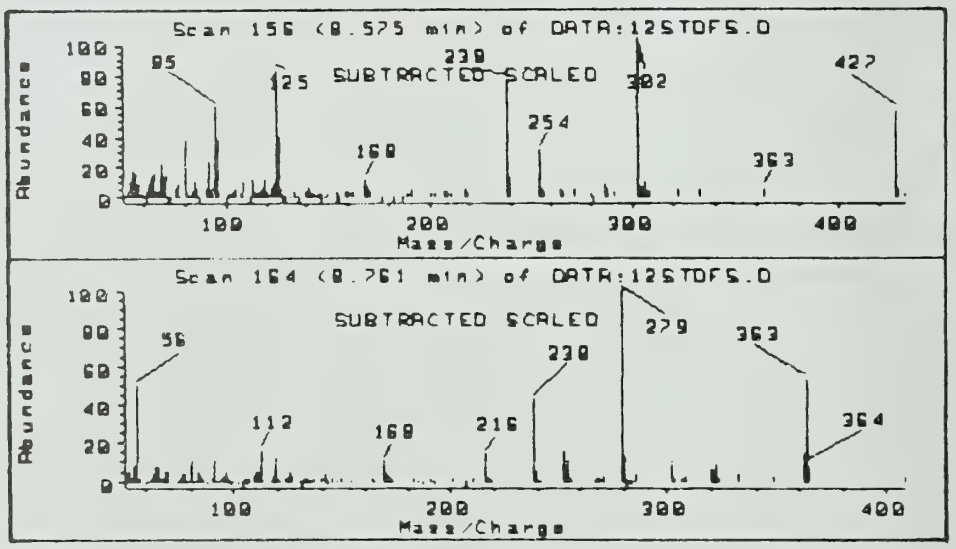
F. ANALYTICAL CONFIRMATION METHOD (Continued)

4. Sample Chromatograms

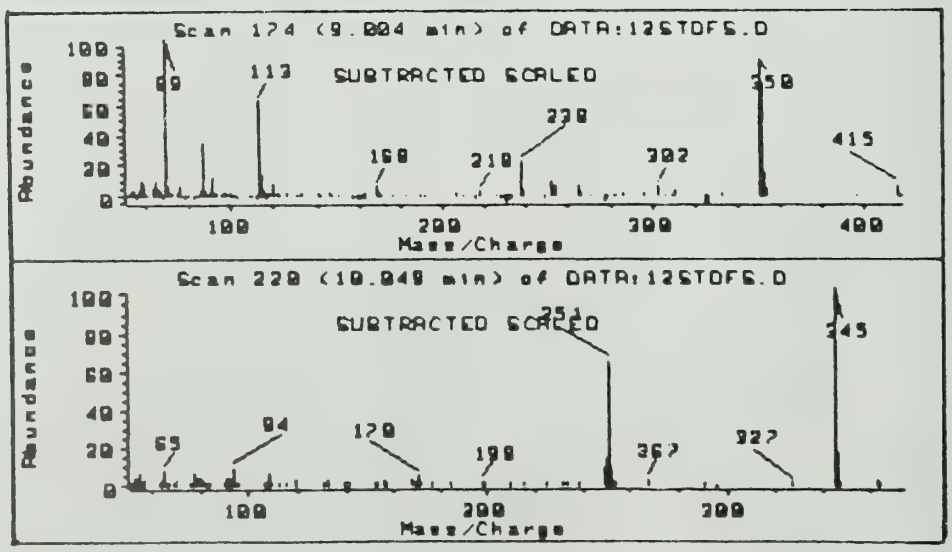
Elution Order:

1. SFX
2. STX
3. STZ and SPY
4. SDZ
5. SRZ
6. SMZ
7. SMP
8. SCP and SEP
9. SDX
10. SDM
11. SPZ
12. SQX





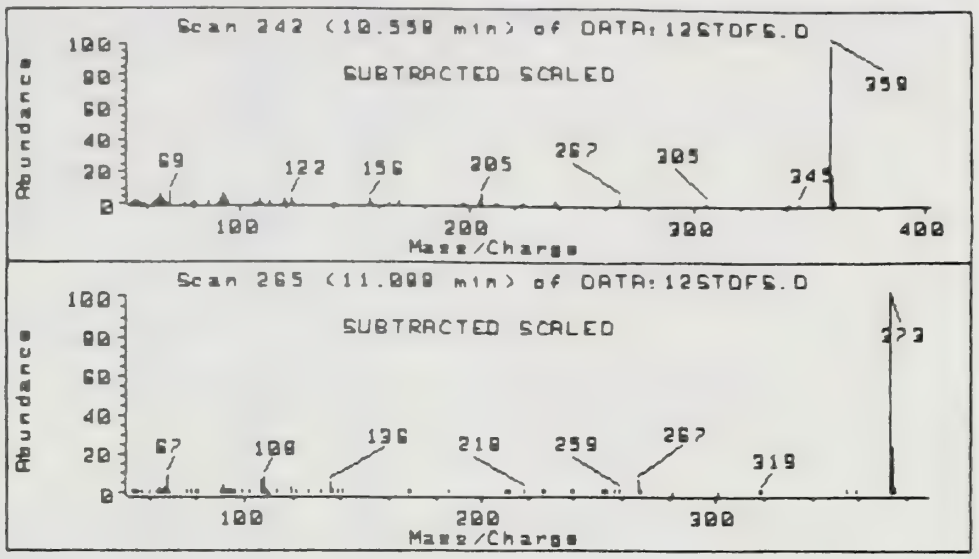
1. Sulfisoxazole
2. Sulfatroxazole



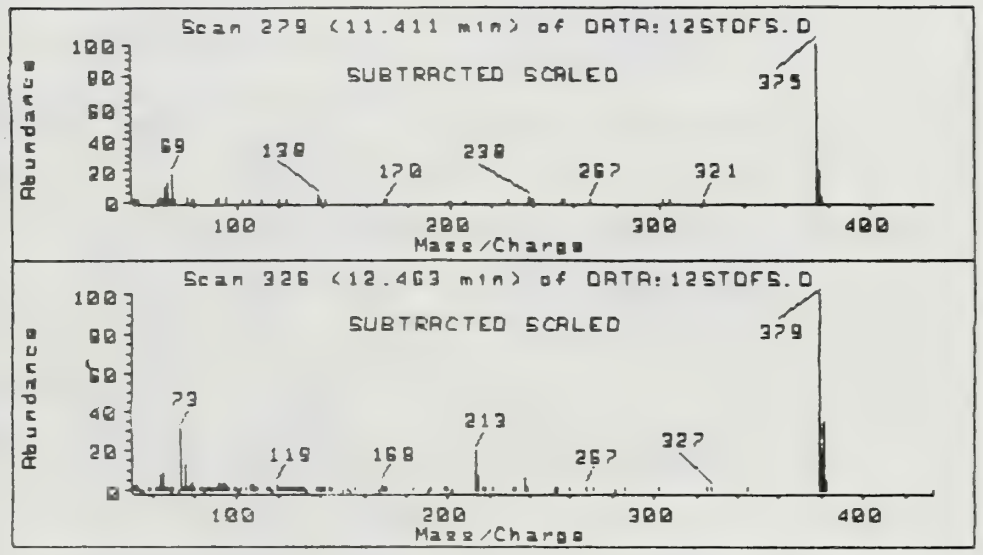
3. Sulfathiazole
4. Sulfadiazine

II. CONFIRMATORY METHOD

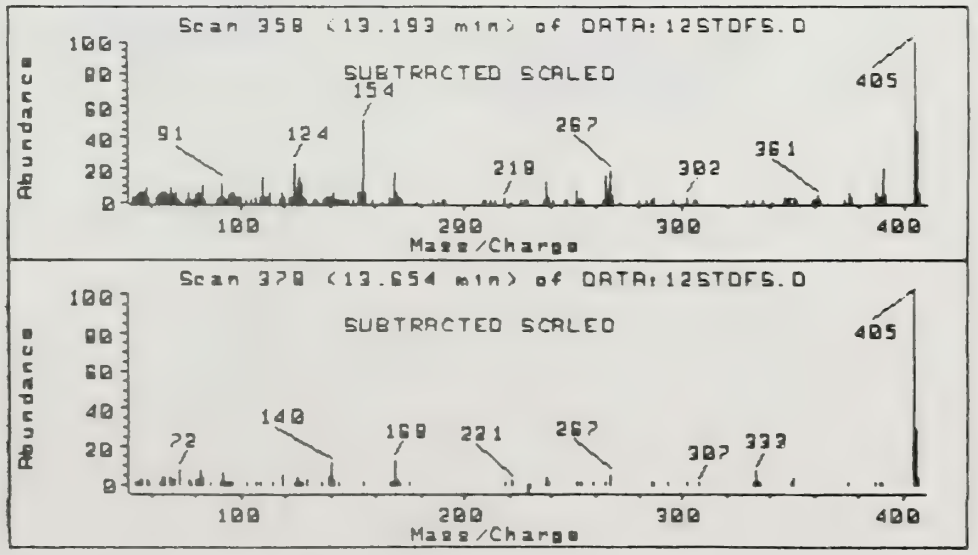
F. ANALTYICAL CONFIRMATION PROCEDURE (Continued)



5. Sulfamerazine
6. Sulfamethazine



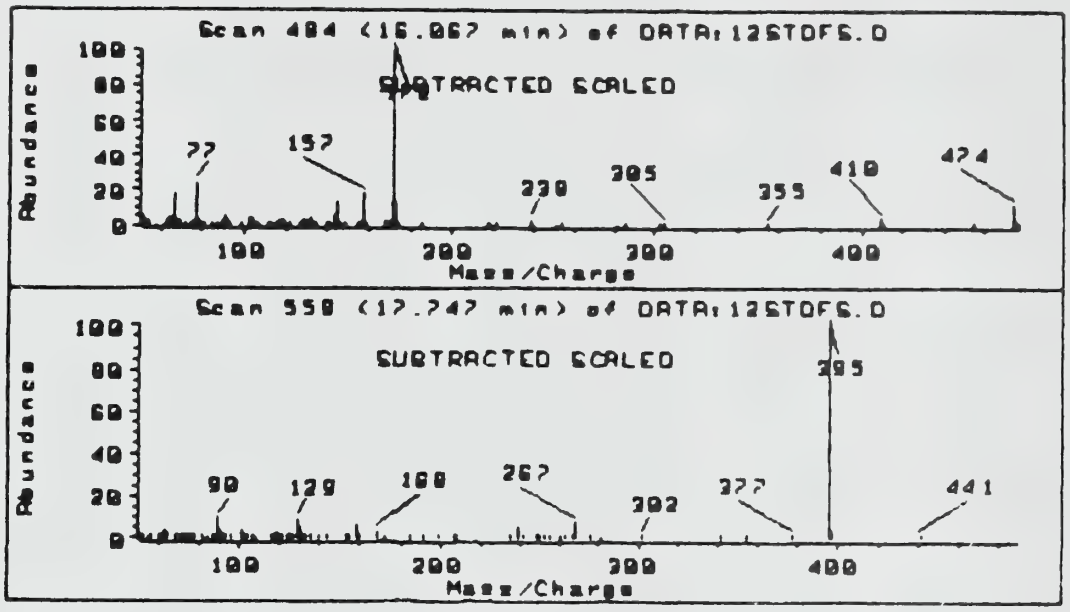
7. Sulfamethoxypyridazine
8. Sulfachloropyridazine



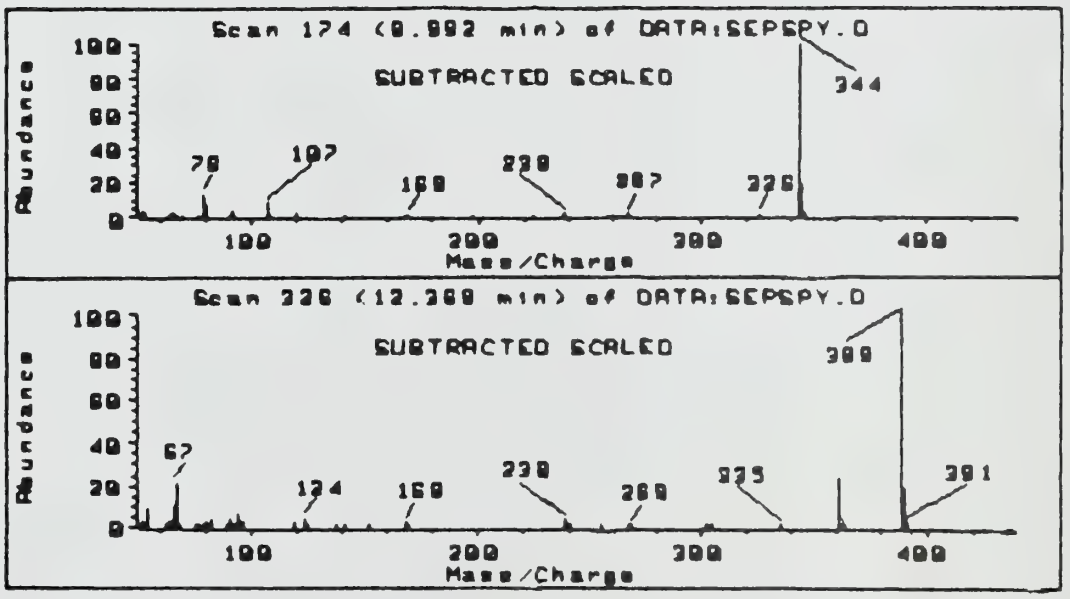
9. Sulfadoxine
10. Sulfadimethoxine

II. CONFIRMATORY METHOD

F. ANALTYICAL CONFIRMATION PROCEDURE (Continued)



11. Sulfaphenazole
12. Sulfaquinoxaline



3. Sulfapyridine
8. Sulfaethoxypyridazine

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

1. Method Title

GC/EI-MS Detection/Confirmation of Sulfonamide Residues in Liver and Muscle Tissue of Swine, Poultry, and Cattle

2. Required Protective Equipment

Safety glasses, plastic gloves, lab coat, shield, and efficient fume hood.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C.8. Diazomethane generation.	Explosion.	Follow generator manufacturer's directions.
N-methyl-N-nitroso-p-toluene sulfonamide.	Skin irritant.	Avoid skin contact.
Ethyl ether.	Detonation.	Check for peroxides before using.
Potassium hydroxide.	Caustic.	Avoid skin contact.
C.8./E.1.d. Diazomethane.	Carcinogen; toxic and explosive.	Keep cold (@ 0° C); use dilute solutions under fume hood and behind shield.
E.1.f. Pentafluoropropionic acid.	Causes severe burns; harmful if swallowed.	Avoid skin contact. Do not swallow.
Diethylamine.	Flammable. Skin irritant.	Perform steps under an efficient hood. Keep from flame, sparks, and heat. Avoid contact with skin and eyes.
Organic solvents.	Flammable.	Perform steps under an efficient hood. Keep from flame, sparks, and heat.

II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS (Continued)

4. Disposal Procedures	Organic solvents.	See above.	Hold in designated containers until disposed of by the contractor or the in-house specialist.
	Byproduct and excess reagents from diazomethane generation.		Flush down in hood drain with large quantities of water.

II. CONFIRMATORY METHOD

I. WORKSHEET

The worksheet on the following page, *Mass Spectrometry Data Report*, can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

MASS SPECTROMETRY DATA FORM

Analyte: _____ Tissue: _____

	R.T. (min) Base Peak	Ion Abundance (m/z)				Ion Ratios		
	_____	_____	_____	_____	_____	_____	_____	_____
Blank								
Standard								
Fortified Tissue								
Sample #								

Concentration:

Standard: _____

Fortified tissue: _____

Sample: _____

For each set of samples of a particular analyte submitted for confirmation, reference standards and blank and fortified tissue samples for the compound of interest must be included. This guideline applies to each analyte on a daily basis.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives from blank tissues.

b. No false negatives at or above 0.1 ppm.

2. Critical Control Points and Specifications

Intraset.

a.	Step II.E.1.d.	Diazomethane < 1 week old or color a deep yellow.
b.	Step II.E.1.d.	Assure excess of diazomethane.
c.	Step II.E.1.d.	Methylation time ≥ 10 minutes.
d.	Step II.E.1.f.	Acylation time ≥ 30 minutes.
e.	Step II.E.1.h.	Remove aqueous layer immediately following centrifugation.

3. Readiness To Perform

a. Phase I.

On two separate days, analyze by selected ion monitoring a 0.1 ppm external standard containing the four sulfonamides normally confirmed (SMZ, SDM, STZ, and SQX). Monitor 238, M-64, and M-65 ions for each sulfonamide. Determine the retention time and the M-64/M-65 ratio for each sulfonamide.

b. Phase II.

Analyst fortified samples. Conduct replicate analyses of control tissue fortifed with 0.10 ppm of the appropriate sulfonamide. It is suggested that liver and muscle tissue from swine, turkey, and bovine species be confirmed with particular emphasis on:

i. Sulfaquinoxaline (SQX), sulfadimethoxine (SDM), and sulfamethazine (SMZ) in turkey.

ii. Sulfamethazine, sulfathiazole (STZ), and sulfadimethoxine in swine.

iii. Sulfamethazine and sulfathiazole in beef.

It is suggested that the above combinations be run twice in turkey liver and muscle, swine liver and muscle, and beef liver and muscle omitting sulfapyridine as internal standard when sulfathiazole is being confirmed. (Total number of fortified samples is twelve.) Also include at least 1 blank for each tissue/species. (Total of six blanks.) Must be able to meet criteria for confirmation in section F.2.

NOTE: Phases I and II may be performed concurrently.

4. Intralaboratory Check Samples

a. Frequency—not determined.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability	See Determinative Method, section J.5.
6. Sample Set	a. Standards. b. Tissue blank. c. Tissue fortified at level of interest with suspect drug. d. Samples.
7. Sensitivity	Sensitivity varies greatly. a. Lowest detectable limit (LDL): Not applicable. b. Lowest reliable confirmation (LRC): 0.05 ppm.

CHLORINATED TRIAZINES IN FAT

Contents

I. Determinative Method

A. Introduction 1
B. Equipment 3
C. Reagents and Solutions 4
D. Standards 5
E. Extraction Procedure 7
F. Analytical Quantitation 10
G. Calculations 14
H. Hazard Analysis 15
I. Worksheet 16
J. Quality Assurance Plan 19

II. Confirmatory Method

A. Introduction 22
B. Equipment 22
C. Reagents 22
D. Standards 22
E. Sample Preparation and Cleanup 23
F. Analytical Confirmation Procedure 24
G. [Reserved]
H. Hazard Analysis 31
I. [Reserved]
J. Quality Assurance Plan 31

I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory and Structures

Chlorinated triazine herbicides are widely used in the production of grain and cereal crops. There is the potential for contamination of feed used in animal production resulting from this use.

The compounds tested are propazine, terbuthylazine, atrazine, and simazine. Triazine compounds are isolated from the sample matrix by extraction with acetonitrile from a solution of rendered fat in petroleum ether.

The acetonitrile extract is evaporated to dryness and the residue dissolved in petroleum ether and eluted through a column of Florisil. Detection of the isolated triazine compounds is accomplished by capillary gas chromatography equipped with nitrogen phosphorous detector (NPD). The compound of interest may be quantitated accurately in the range of 10-40 ppb.

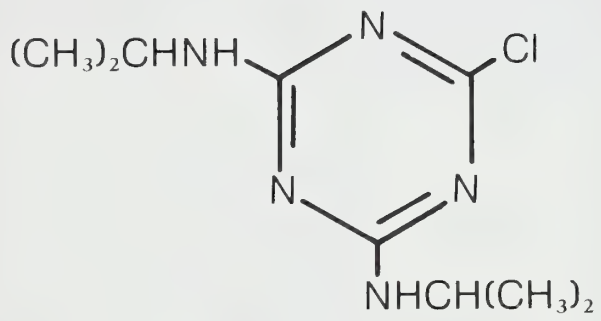
Propazine	$C_9H_{16}ClN_5$ F.W. = 229.7
Terbuthylazine	$C_9H_{16}ClN_5$ F.W. = 229.7
Atrazine	$C_8H_{14}ClN_5$ F.W. = 215.7
Simazine	$C_7H_{12}ClN_5$ F.W. = 201.7

2. Applicability

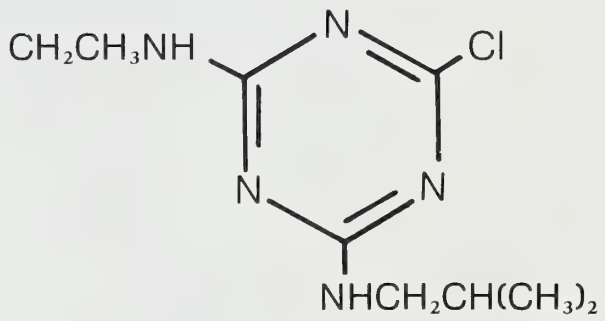
This method has been evaluated for triazine analysis in the fat of bovine, porcine, and avian species.

I. DETERMINATIVE METHOD

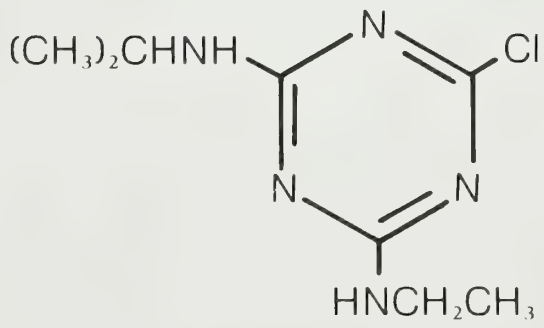
A. INTRODUCTION (Continued)



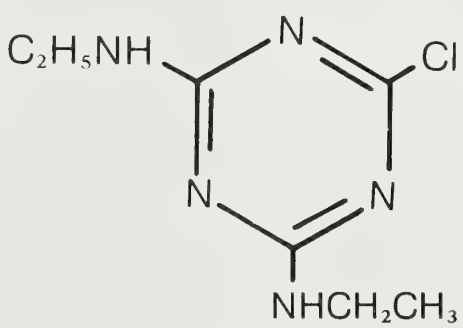
PROPAZINE



TERBUTHYLAZINE



ATRAZINE



SIMAZINE

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

An equivalent can be substituted for any apparatus listed below if necessary.

a. 50 mL glass centrifuge tubes (Pyrex #8240) or polypropylene centrifuge tubes (Corning #25330).

b. 15 mL glass centrifuge tubes (Fisher #05-538-20 A).

c. 60 mL separatory funnel.

d. Pipet dispensers.

e. N-Evap solvent evaporation unit (Organomation, Inc.).

f. Volumetric flasks: 25 mL, 100 mL.

g. Vortex mixer.

h. Florisil columns: glass or polypropylene column with the following characteristics:

   i. Inside diameter 1.0 to 1.1 cm.

   ii. Length at least 4 cm.

   iii. Reservoir at least 10 mL.

NOTE: The columns used for this work are Fisher Prep-Sep for Robotic applications. These columns were purchased prepacked with Florisil that was found not to be acceptable for this procedure. However, the columns were ideal for this work, so the commercial packing was discarded and the empty columns were repacked with Florisil prepared in-house. Any column of suitable dimensions should work for this method. An example of an acceptable column would be Alltech Assoc. (#17312).

2. Instrumentation

a. Hewlett Packard model 5890 gas chromatograph equipped with NPD nitrogen-phosphorous detection system, automatic sampler, split/splitless capillary injection, and appropriate data logging or recording device.

b. Capillary column: 30 m, 0.32 mm id fused silica capillary column coated with Supelcowax 10 (0.25 μm film thickness) liquid phase (Supelco, Inc. #2-4080).

c. Oxygen scrubber: Alltech Associates Oxy Trap or equivalent.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Acetonitrile, petroleum ether, diethyl ether, nonane, methanol, acetone: All solvents should be pesticide grade or better and produce acceptable response for reagent blank (See section F).

b. Diethyl ether:petroleum ether (6:94): Measure 60 mL diethyl ether into a 1000 mL volumetric flask and dilute to volume with petroleum ether.

c. Diethyl ether:petroleum ether (15:85): Measure 150 mL diethyl ether into a 1000 mL volumetric flask and dilute to volume with petroleum ether.

d. Florisil: (Alltech Assoc. #5618) Pesticide grade (100/200 mesh); activate for 4 hours at 600° C and store at 110° C until used.

Florisil is deactivated by adding water (10% by weight) 24 hours prior to use. Mixture should be shaken gently to distribute the water and stored overnight in a tightly stoppered flask. Columns should be packed immediately before use since deactivation level may change depending on ambient humidity.

I. DETERMINATIVE METHOD

D. STANDARDS

1. Source

Each new lot of standard should be prepared as stated below and compared to current standards. All triazine standards are to be procured from:

Director
Environmental Monitoring Support Laboratory
Office of Research & Development
Cincinnati, OH 45268

Propazine (#5800)
Terbuthylazine (#6589)
Atrazine (#0420)
Simazine (#6160)
Cyprazine—internal standard (#1615)

2. Preparation of Standards

a. Stock standards (1.0 mg/mL)—Accurately weigh 25.0 mg of each triazine reference standard into a clean 25 mL volumetric flask. Dissolve and dilute to volume with methanol except for simazine, which is dissolved in dimethylformamide.

b. Working standards (10 μg/mL)—Pipet 1.0 mL of each triazine stock standard solution into a separate clean 100 mL volumetric flask and dilute to volume with iso-octane.

c. Fortification standards

i. 40 ppb spiking solution = 0.8 μg/mL triazines
0.4 μg/mL cyprazine

Pipet 8.0 mL of the 10 μg/mL solution of propazine, terbuthylazine, atrazine, and simazine and 4.0 mL of the 10 μg/mL solution of cyprazine into a clean 100 mL volumetric flask and dilute to volume with iso-octane.

ii. 20 ppb spiking solution = 0.4 μg/mL triazines
0.4 μg/mL cyprazine

Pipet 4.0 μL of the 10 μg/mL solution of propazine, terbuthylazine, atrazine, and simazine and 4.0 mL of cyprazine into a clean 100 mL volumetric flask and dilute to volume with iso-octane.

iii. 10 ppb spiking solution = 0.2 μg/mL triazines
0.4 μg/mL cyprazine

Pipet 2.0 mL of the 10 μg/mL solution of propazine, terbuthylazine, atrazine, and simazine into a clean 100 mL volumetric flask and dilute to volume with iso-octane.

iv. Internal standard spiking solution = 0.4 μg/mL

Pipet 4.0 mL of cyprazine only into a clean 100 mL volumetric flask and dilute to volume with iso-octane.

I. DETERMINATIVE METHOD

D. STANDARDS (Continued)

d. Gas Chromatography Standards

All ''ppb GC standards'' are calculated using 500 μL as sample final volume and 2.0 g as sample weight.

i. 40 ppb GC standard = 160 ng/mL triazines
80 ng/mL cyprazine

Pipet 2.0 mL of the ''40 ppb spiking standard'' into a clean 10 mL volumetric flask and dilute to volume with nonane.

ii. 20 ppb GC standard = 80 ng/mL triazines
80 ng/mL cyprazine

Pipet 2.0 mL of the ''20 ppb spiking standard'' into a clean 10 mL volumetric flask and dilute to volume with nonane.

iii. 10 ppb GC standard = 40 ng/mL triazines
80 ng/mL cyprazine

Pipet 2.0 mL of the ''10 ppb spiking standard'' into a clean 10 mL volumetric flask and dilute to volume with nonane.

3. Storage Conditions

All standards should be kept tightly closed and refrigerated at 4°C when not in use.

4. Shelf Life Stability

a. Stock, working, and fortification standards are stable for periods up to 6 months once prepared when stored under the above conditions.

b. GC standards are stable for periods up to 4 weeks once prepared when stored under the above conditions.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Sample Preparation

Pass chilled fat sample through food chopper twice. If dry ice is available, add small quantities to the fat to maintain a low temperature. Plug a powder funnel moderately tight with glass wool; place approximately 100 g of the contaminated fat in the funnel in a beaker and render at 100-110° C until the fat ceases to drop.

2. Column Preparation

Prepare a Florisil column for each sample by weighing 1.0 g of deactivated Florisil into a glass or polypropylene chromatography column. Prewet column with 10 mL petroleum ether, allow solvent to drain completely, and discard wash solvent. Do not allow columns to go dry. Columns are ready to be used for step 3.i below.

3. Sample Extraction

a. Weigh 2.0 g of rendered fat into a clean 50 mL centrifuge tube. Select a blank sample and weigh four separate 2.0 g samples. Label these samples and fortify them as follows:

   i. ''Blank'' fortified with 100 μL of ''internal standard.''

   ii. ''10 ppb standard'' fortified with 100 μL of ''10 ppb spiking standard.''

   iii. ''20 ppb standard'' fortified with 100 μL of ''20 ppb spiking standard.''

   iv. ''40 ppb standard'' fortified with 100 μL of ''40 ppb spiking standard.''

b. Fortify the sample with the internal standard as in a.i above.

c. Add 10 mL of petroleum ether to each sample. Mix, using a vortex mixer to dissolve the sample, and transfer to a clean 60 mL separatory funnel. Repeat with two additional washes of 10 mL of petroleum ether, combining all three fractions in the 60 mL separatory funnel.

d. Add 10 mL acetonitrile to each separatory funnel and extract by shaking gently for 1 min. Allow the phases to separate for 5 min.

e. Collect the acetonitrile in a clean 50 mL centrifuge tube. Place the tube containing acetonitrile on a N-Evap solvent evaporation unit and remove the solvent under a stream of dry nitrogen at a temperature of 50-60° C.

f. Repeat the acetonitrile extractions three more times (total of four extractions), combining all in the original 50 mL centrifuge tube as evaporation proceeds and the tube volume permits.

g. After the last acetonitrile extraction has been added to the 50 mL tube, continue solvent evaporation until approximately 8-10 mL of acetonitrile remain. Remove tubes from evaporation unit and add 10 mL petroleum ether. Shake gently for 1 min and allow phases to separate for 5 min. Remove and discard petroleum ether fraction. Place tubes back on evaporation unit and evaporate remaining acetonitrile until tubes are just dry.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

h. Redissolve the oily residue in 5 mL petroleum ether, using a vortex mixer to assure complete dissolution.

i. Transfer each sample to a separate Florisil column (as prepared in section C.2) and allow to flow under gravity, taking care that the columns do not go dry.

j. Wash each column with an additional 10 mL petroleum ether followed by 10 mL of (6:94) ethyl ether:petroleum ether solution. Discard all washes.

k. Place a clean 15 mL centrifuge tube beneath each column and elute the triazine compounds with 15 mL of (15:85) ethyl ether:petroleum ether.

l. Place column eluates on N-Evap unit and carefully remove all solvent under a stream of dry nitrogen, using a bath temperature of 40-45° C. Do not allow the tubes to remain on the evaporation unit after solvent has completely evaporated.

m. Dissolve residue in 500 μL of a mixture of 1:5 iso-octane:nonane.

n. Transfer extracts to auto sampler vials for analysis by capillary GC operated in the splitless injection mode.

3. Florisil Evaluation

New batches of Florisil should be evaluated for suitable performance in this method in the following manner:

a. Prepare column of new material in normal manner.

b. Add 100 μL of ''40 ppb spiking standard'' to the column.

c. Wash with 10 mL petroleum ether and discard.

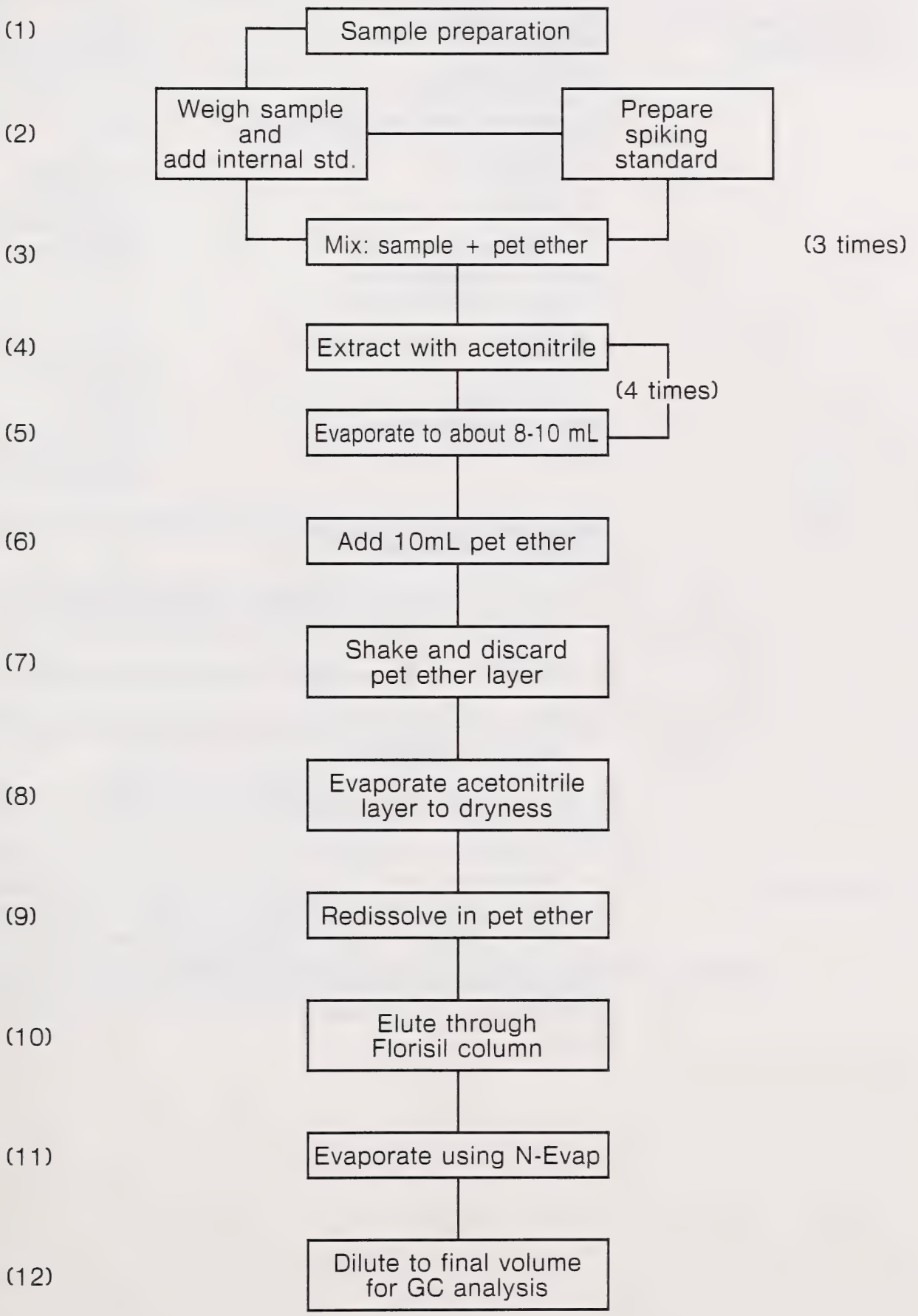
d. Elute with 10 mL of 6:94 ethyl ether:petroleum ether and collect. Evaporate the solvent, redissolve in 500 μL of 1:5 iso-octane:nonane, and analyze by capillary GC. If propazine is found, it is an indication that the water is too high. Deactivate with lower percentage of water or store at 130° C instead of 110° C.

e. Elute column above with 15 mL of (15:85) ethyl ether:petroleum ether and collect. Evaporate the solvent, redissolve the eluate in 500 μL of (1:5) iso-octane:nonane, and analyze by capillary GC. If simazine recovery is $< 75\%$, the Florisil is not deactivated sufficiently. Increase percentage of water used for deactivation.

I. DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

4. Flow Chart Summary



I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Instrumental Settings and Conditions

Backflush time	1.0 min
Attenuation	4 (depends on detector sensitivity)
Chart speed	0.5 cm/min
Injection volume	1 μL
Carrier gas and make-up gas	Helium
Flow rate:	
Carrier gas	1.2 mL/min
Makeup	20 mL/min
Air	100-120 mL/min
Hydrogen	3.5 mL/min
Detector temperature	275° C
Column temperature:	
Initial	130° C
Hold time	2 min
Program rate	40° C/min
Final temp	210° C
Injection temperature	195° C
Column used	Fused silica capillary coated with Supelcowax 10 (30 m × 0.32mm)

NOTE: Lifetime of the GC column is greatly extended by adhering to the following operating principles:

a. Use an oxygen scrubber in carrier gas at all times. Supelcowax 10 is sensitive to trace levels of oxygen, especially at elevated temperature.

b. Do not change septum if column temperature is above 50° C.

c. Keep column and injector temperature below 50° C for 30 minutes after septum is changed.

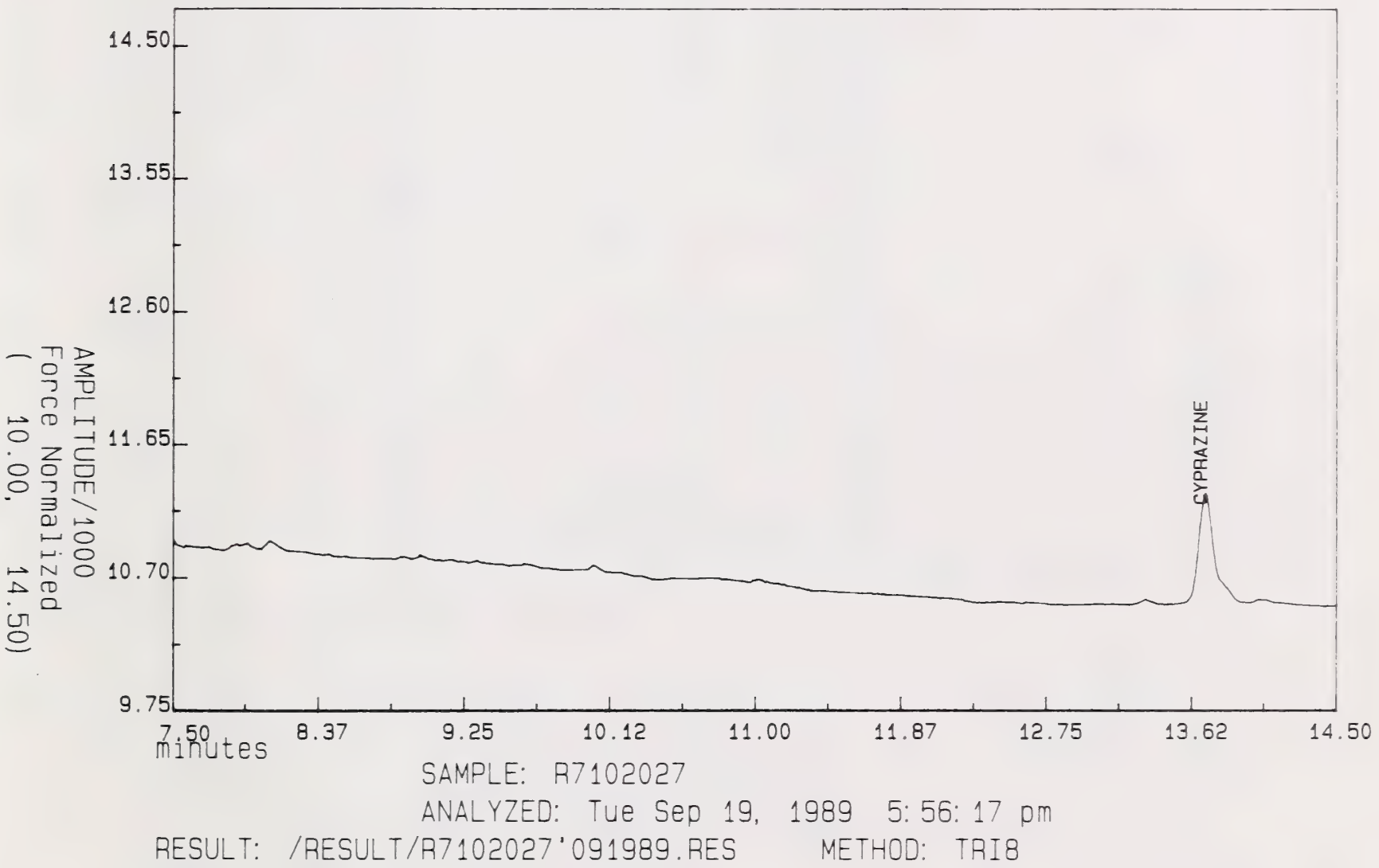
Failure to follow these guidelines may result in rapid or instantaneous column failure.

2. Interferences

Chromatograms of triazine extracts are generally clear of extraneous peaks that could interfere with quantitation of triazine compounds. Occasional peaks may appear at retention times sufficiently different from triazine compounds to make identification of the triazines uncomplicated. Hewlett Packard NPD detector responds with a negative signal to high levels of hydrocarbon materials. Occasional negative peaks may be encountered, especially with pork and beef matrix.

I. DETERMINATIVE METHOD

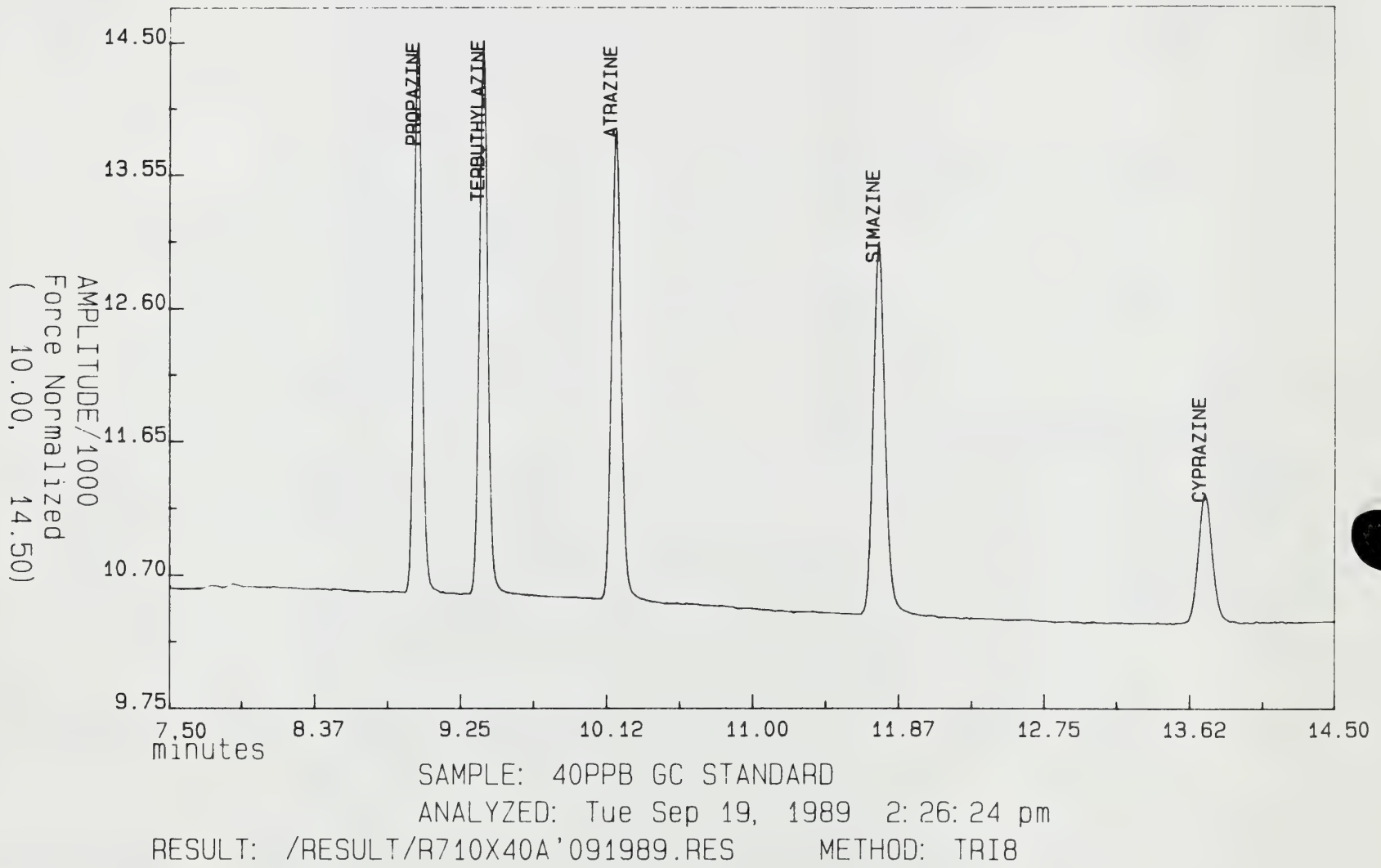
F. ANALYTICAL QUANTITATION (Continued)



Tissue blank fortified with 80 ppb internal standard.

I. DETERMINATIVE METHOD

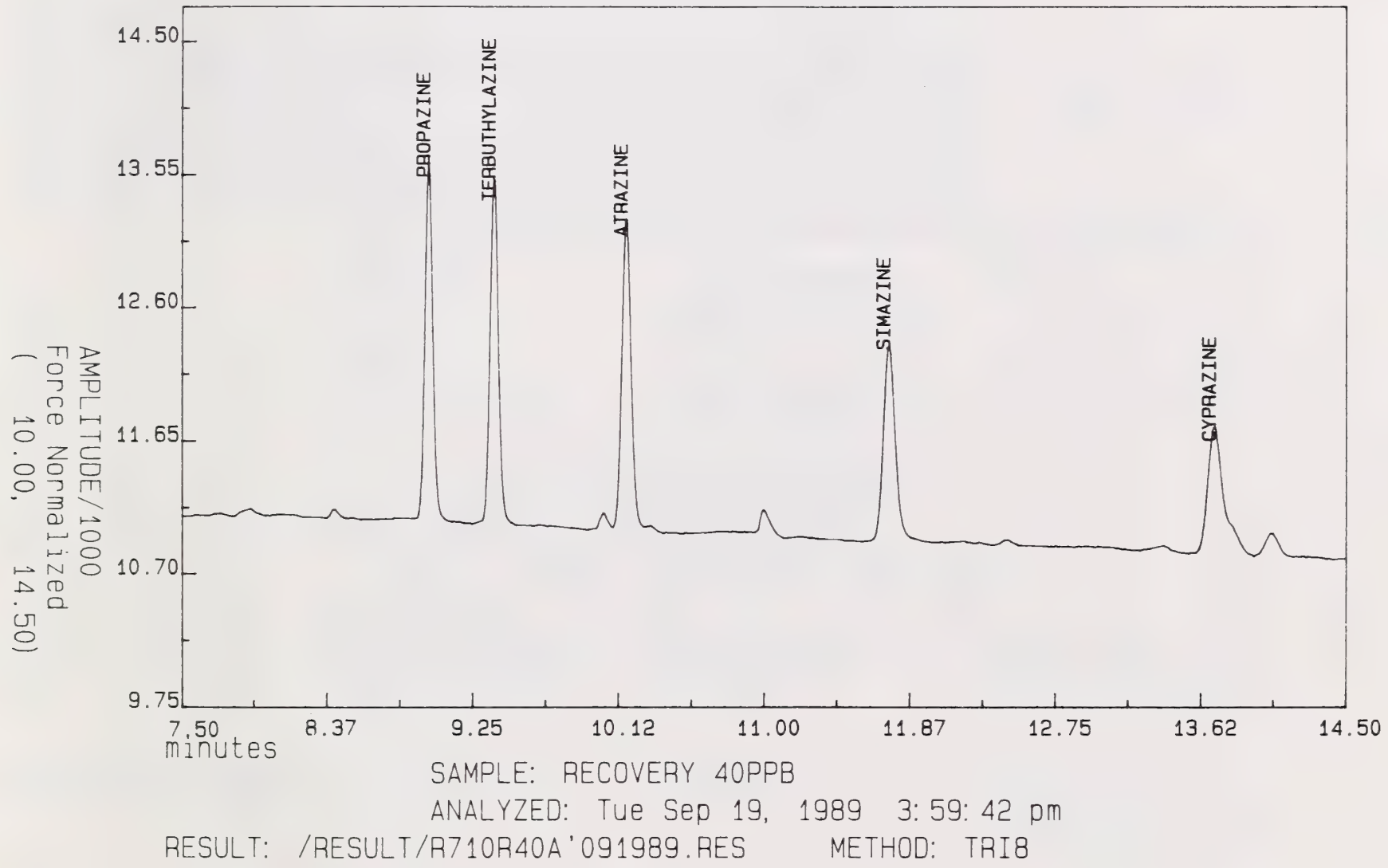
F. ANALYTICAL QUANTITATION (Continued)



Triazines standard diluted in nonane (40 ppb).

I. DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)



Blank tissue spiked with 40 ppb triazines standard and 80 ppb internal standard.

I. DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Cyprazine is used as an internal standard for calculating component concentration. Proceed in the following manner to calculate linear calibration curves and component concentrations.

By an acceptable mean, measure the peak area for each component in the 0, 10, 20, and 40 ppb spiked samples that have been processed through the procedure. Calculate the peak area ratios for each triazine compound by dividing the individual triazine peak area by the cyprazine peak area. Using the ratios and associated ppb values, calculate a linear calibration curve by least squares computation as indicated in the Chemistry Quality Assurance Handbook, Vol. II, Section 1.5.67. These calibration curves are then used to calculate values for additional samples from the sample set.

$y = mx + b$

where

m = slope

b = intercept

x = triazine(s) area count/cyprazine area count

y = triazine concentration in ppb (ppb = ng/g, using 500 μL as sample volume and 2.0 g as sample weight)

Standard curve correlation coefficient $\geq$ 0.990.

Refer to section J for additional acceptability criteria.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title — Analysis of Chlorinated Triazines

2. Required Protective Equipment — Safety glasses, plastic gloves, lab coat.

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Petroleum Ether Acetonitrile Ethyl Ether Toluene Iso-octane Nonane Dimethylformamide	Highly flammable. Explosive hazard. Vapors mixed with air will explode if ignited. Irritating to skin and mucous membrane. Inhalation of high concentration will cause narcosis, unconsciousness.	Keep tightly closed in a cold place and away from fire. Avoid breathing vapor. Use under fume hood.
E. Extraction		
Evaporation using N-Evap and water bath	May result in explosion.	Check glassware for flaws and work behind a protective shield.

4. Disposal Procedures — Organic solvents | See above | Dispose of in proper container and segregate chlorinated from nonchlorinated.

I. DETERMINATIVE METHOD

I. WORKSHEET

The worksheet on the facing page, *Triazine Residues in Beef, Pork, and Poultry Fat,* can be removed from this book for photocopying whenever necessary, but do not forget to replace it.

TRIAZINE RESIDUES IN BEEF, PORK, AND POULTRY FAT

INSTRUMENT CONDITIONS

ANALYST: _____

Date Started: _____

Date Completed: _____

Column: Supelcowax 10, 30 m × 0.32 mm I.D., 0.25 μm D.F.
Oven Program: 130° C for 2 min, heat 40° C/min to 210° C, hold for 15 min.

Injector: 195° C
Detector: 275° C
Volume: 1 μL
Chart Speed: 0.5 cm/min
Attenuation: 4 mV/FS
Integration: HP-LAS

Carrier: He 1.2 cc/min
Septum Vent: 3.0 cc/min
Split Vent: 80 cc/min
Make-Up Gas: 20 cc/min
Hydrogen: 3.5 cc/min
Air: 120 cc/min

SAMPLE ANALYSIS DATA

Sample Identification	Species	Weight g ± 0.1g	Final Volume μL	Cyprazine Std. ppb	Propazine ppb	Terbuthylazine ppb	Atrazine ppb	Simazine ppb

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound List	*Analytical Range (ppb)*	*Acceptable Recovery %*	*Repeatability %CV_o*
Propazine	5-40	80-110	≤ 15 at 20 ppb
Terbuthylazine	5-40	80-110	≤ 15 at 20 ppb
Atrazine	5-40	80-110	≤ 15 at 20 ppb
Simazine	5-40	80-110	≤ 15 at 20 ppb

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	2.0 ± 0.1 g
b. Florisil weight	1.0 ± 0.05 g
c. Elution volume	15 ± 1.0 mL
d. Final extract volume	500 μL ± 10 μL

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—Duplicate standard curve on each of 3 consecutive days at 0, 10, 20, and 40 ppb for each of the four triazines.

ii. Phase II: Self-fortified samples—Duplicate analyses on fortified samples at 0, 10, 20, and 40 ppb on 3 consecutive days, one set each of beef, pork, and poultry fat with all four triazines.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst's accreditation.

(a) 12 samples submitted by the supervisor.

(i) Fat tissue for all 3 species (beef, pork, and poultry), 4 of each kind.

(ii) Samples fortified at 10-40 ppb.

(iii) Each sample must contain at least one of the triazines.

(b) Report data to Chemistry Division, QSB.

b. Acceptability criteria.

Refer to section J.1 above.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents:

i. Frequency: At least one check sample biweekly per analyst containing at least one of the triazines and using different species. At least one out of four of the positive samples is to be confirmed by mass spectrometry.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) Unknown check samples.

(i) All findings, noting all replicates.

(ii) Control chart on percent difference between replicates (CUSUMs).

(b) Known recovery samples.

(i) Separate record for each analyte/species.

(ii) % recoveries for each set using 10-day running averages.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

iv. Repeat Phase III of section J.3 above if cause was analyst-related.

I. DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability

a. Matrix: Fat.

b. Sample receipt size, minimum: 50 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage.

i. Time: 6 months.

ii. Condition: −20° C.

6. Sample Set

a. Screening method.

For screening purposes, a sample set can include a mixture of all three different species with a tissue blank, a tissue blank fortified at 20 ppb, and up to 12 additional samples per set.

b. Quantitation for screened positives.

i. For any sample found positive in 6.a, each set must contain:

(a) A blank tissue fortified with the internal standard at 80 ppb.

(b) A 10 ppb fortified triazines standard.

(c) A 20 ppb fortified triazines standard.

(d) A 40 ppb fortified triazines standard.

(e) Up to 12 additional positive samples.

ii. Refer to Section D for more details on standard preparation.

iii. Blank tissue used for the standards and blank as described above must be of the same species as the positive sample.

7. Sensitivity

a. Lowest detectable level (LDL): 5 ppb.

b. Lowest reliable quantitation (LRQ): 10 ppb.

c. Minimum proficiency level (MPL): Not determined.

II. CONFIRMATORY METHOD

A. INTRODUCTION

Refer to Determinative Method, section A.

B. EQUIPMENT

1. Apparatus

Refer to Determinative Method, section B.1.

2. Instrumentation

GC/MS: HP5890/5970 Mass Spectrometer—Ion Selective Detector/ Capillary Direct Interface

C. REAGENTS

All reagents as in Determinative Method, section C, in addition to toluene (pesticide quality).

D. STANDARDS

Procedure

Working standard = 1 μg/mL.

Using the working standards prepared in Determinative Method, section D.2, dilute 1 mL of all appropriate standards (cyprazine and compounds to be confirmed) into a 10 mL volumetric flask and dilute to volume with toluene.

If more concentrated solutions are needed for adequate GC/MS response, use larger aliquots.

II. CONFIRMATORY METHOD

E. SAMPLE PREPARATION AND CLEANUP

Procedure	Sample preparation for mass spectrometric confirmation: Use Determinative Method, section E, to extract and clean up the confirmation sample, a reagent blank, a blank tissue fortified at 20 ppb, and a tissue blank. Evaporate the florisil column eluate under dry nitrogen on an N-evap (set water bath at 40° C) just long enough to remove all traces of volatile solvent. A pungent, oily residue may remain. Sample losses will occur if the tubes are over-dried. Dissolve the residue in a suitable volume of toluene such that the concentration of the analyte is approximately the same as that of the standard. For example, if standard concentration is 1 μg/mL and the sample contains 20 ppb of the compound of interest, the final volume should be 30-40 μL.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE

1. Data Acquisition

GC/MS instrument: HP 5890/5970 Mass Spec-Ion Selective Detector/ Capillary-Direct Interface.

Column: HP-1 capillary.

Liquid phase: Crosslinked methyl silicone, 0.30 μm thick.

Column length: 12 m.

Column diameter: 0.20 mm.

GC conditions:

Carrier gas	Helium
Injection mode	Grob Splitless
Purge flow	40 mL/min
Purge off time	0.6 min
Injection temperature	200° C.
Column head pressure	2.5 psi
Interface temperature	270° C.

Column temperature program

Temp 1	85° C; hold for 1 min
Temp Rate 1	30°/min
Temp 2	175° C
Temp Rate 2	5°/min
Temp 3	195° C
Temp Rate 3	30°/min
Temp 4	250° C; hold for 8 min

MS SIM acquisition parameters

Start time	5.5 min
Ion masses	See Table 1
Dwell time	50 milliseconds/ion
EM voltage	Autotune voltage +200 V
Stop time	7.5 min

NOTE: Temperature program should be adjusted to elute simazine, atrazine, propazine, and terbuthylazine during period of 5°/min temperature ramping.

General requirements:

The GC/MS system must be shown capable of resolving all triazines injecting and detecting them with sufficient sensitivity to permit confirmation when 1-2 ng of analyte are injected. Care must be taken to assure that the GC inlet and column are not retaining analytes. The mass spectrometer should be tuned prior to running confirmatory samples.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

2. Instrument Performance Verification

Set up instrument as described in section F.1 above to monitor all compounds of interest, including the internal standard to verify method recovery. Inject 1-1.5 μL of the external standard and analyze results to verify that the system is functioning properly. If not, make any necessary adjustments in operating parameters or standard concentration, then re-inject to verify performance.

3. Criteria for Confirmation

i. Retention time specification.

Compound	*Approximate Retention Time (min)*	*Relative Retention Time (min)*
Simazine	6.52	0.84
Atrazine	6.65	0.86
Propazine	6.76	0.87
Terbuthylazine	6.97	0.90
Cyprazine	7.75	1.00

Retention time of analyte peaks in the confirmation sample must match the retention time window of the standard or recovery (within 2%).

ii. Number of specific required ions.

Compound	*Ions Monitored (+ = Molecular Ion)*
Simazine	145, 158, 173, 186, 201+, 203
Atrazine	145, 172, 173, 200, 215+, 217
Propazine	145, 172, 187, 214, 229+, 231
Terbuthylazine	145, 172, 173, 214, 229+, 231
Cyprazine	170, 212, 214, 226, 227+, 229

NOTE: Monitor 6 ions for confirmation. Ion 158 will provide slightly higher relative abundance than 145 for all compounds except propazine. If ions 145 or 158 cannot be detected with sufficient sensitivity, substitute the ions listed in Group 7. (Refer to Table 1 on the following page.)

iii. Structure of Confirming Ions

Refer to TRZ-27, 28.

4. Known Interferences

Total ion chromatograms for beef, pork, and poultry fat show no interfering peaks present.

II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

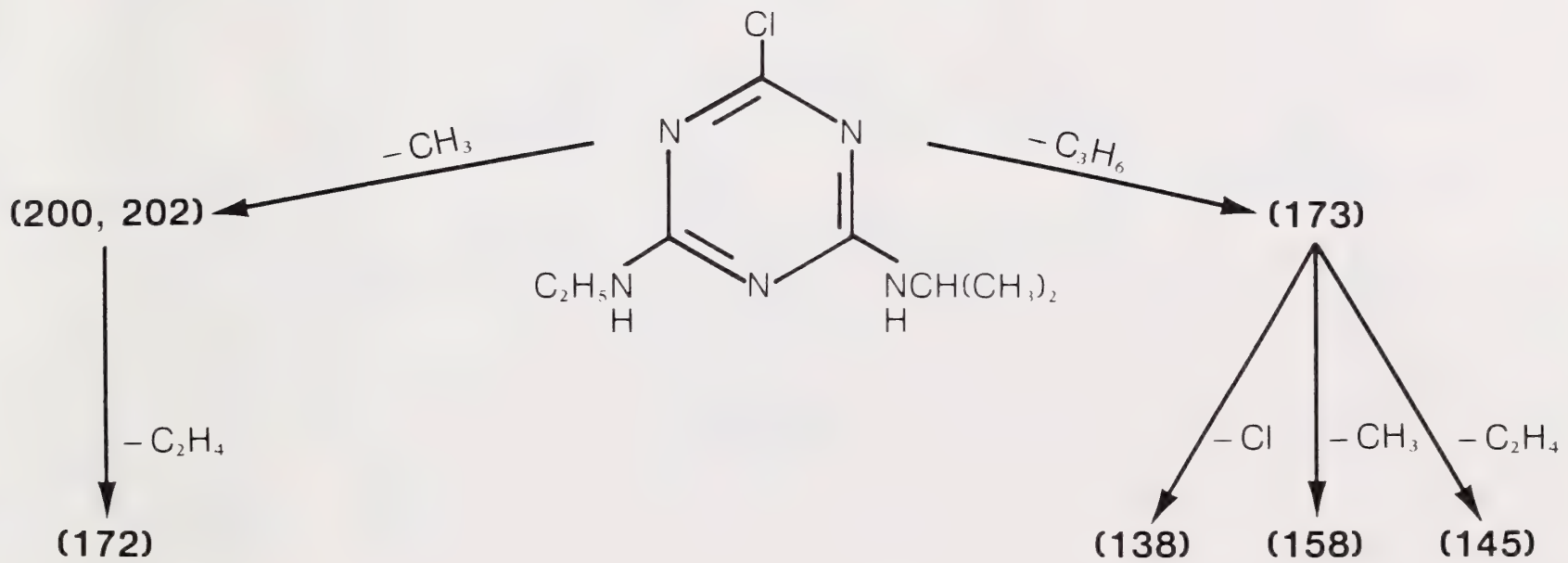
TABLE 1. SIM and Retention Time Data

Compound	*Rt. Time*	*Monitored Ions/Relative Abundance*						
		1	*2*	*3*	*4*	*5*	*6*	*7*
Simazine	6.52	145 .13	158 .22	173 .42	186 .58	201 + 1.0	203 .32	188 .18
Atrazine	6.65	145 .11	172 .16	173 .27	200 1.0	215 + .66	217 .22	202 .34
Propazine	6.76	145 .09	172 .61	187 .27	214 1.0	229 + .71	231 .23	216 .34
Terbuthylazine	6.97	145 .08	172 .12	173 .42	214 1.0	229 + .34	231 .11	216 .34
Cyprazine (ISTD)	7.75	170 .45	212 1.0	214 .33	226 .30	227 .50	229 .17	

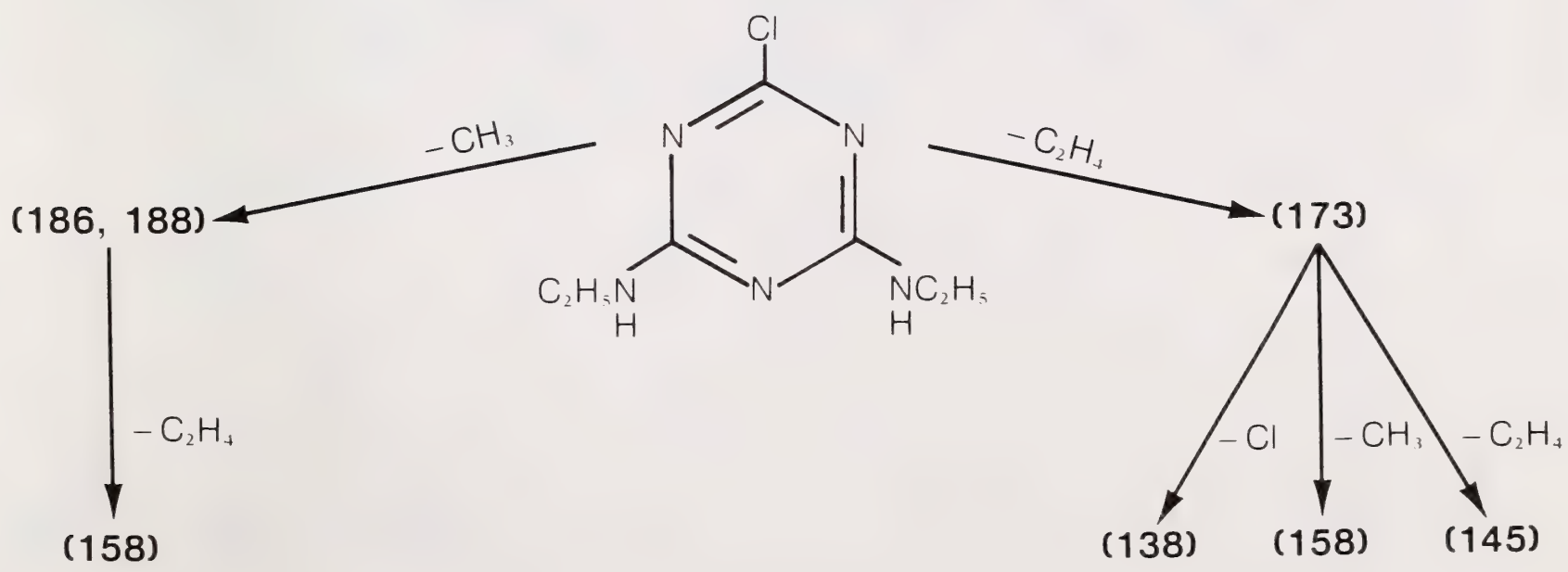
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

ATRAZINE
(215, 217)



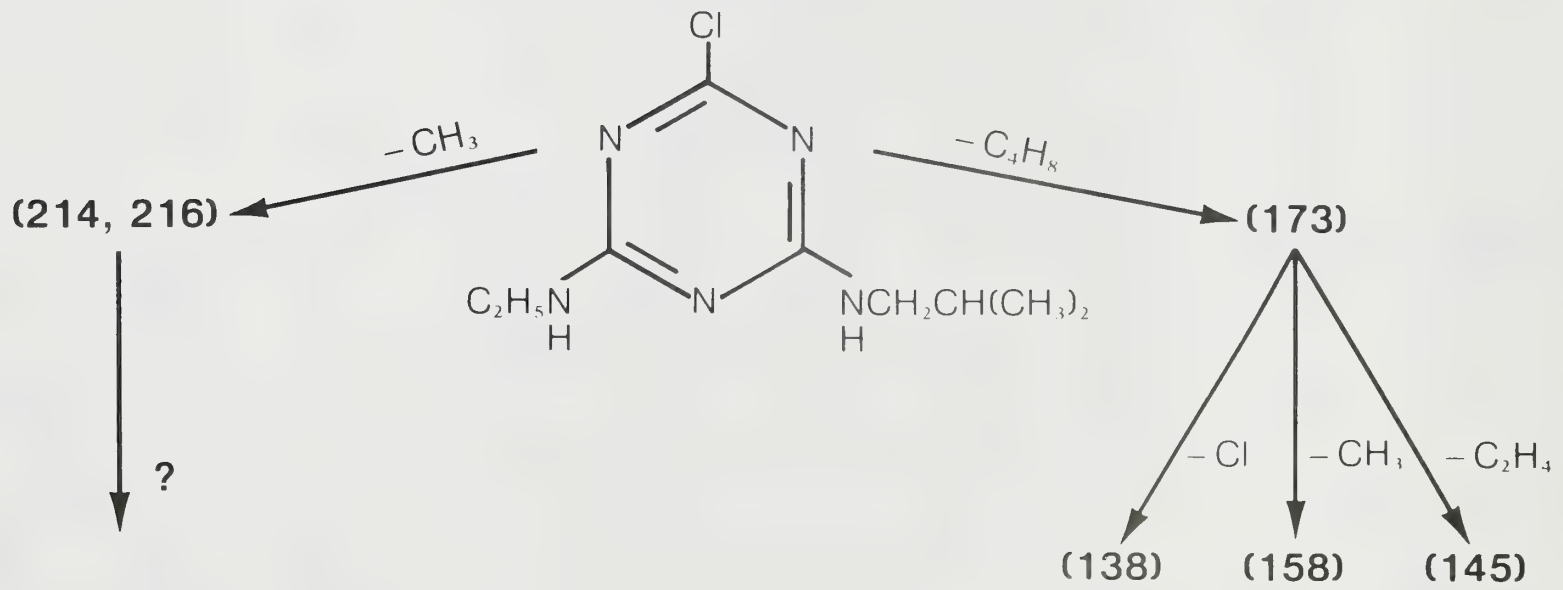
SIMAZINE
(201, 203)



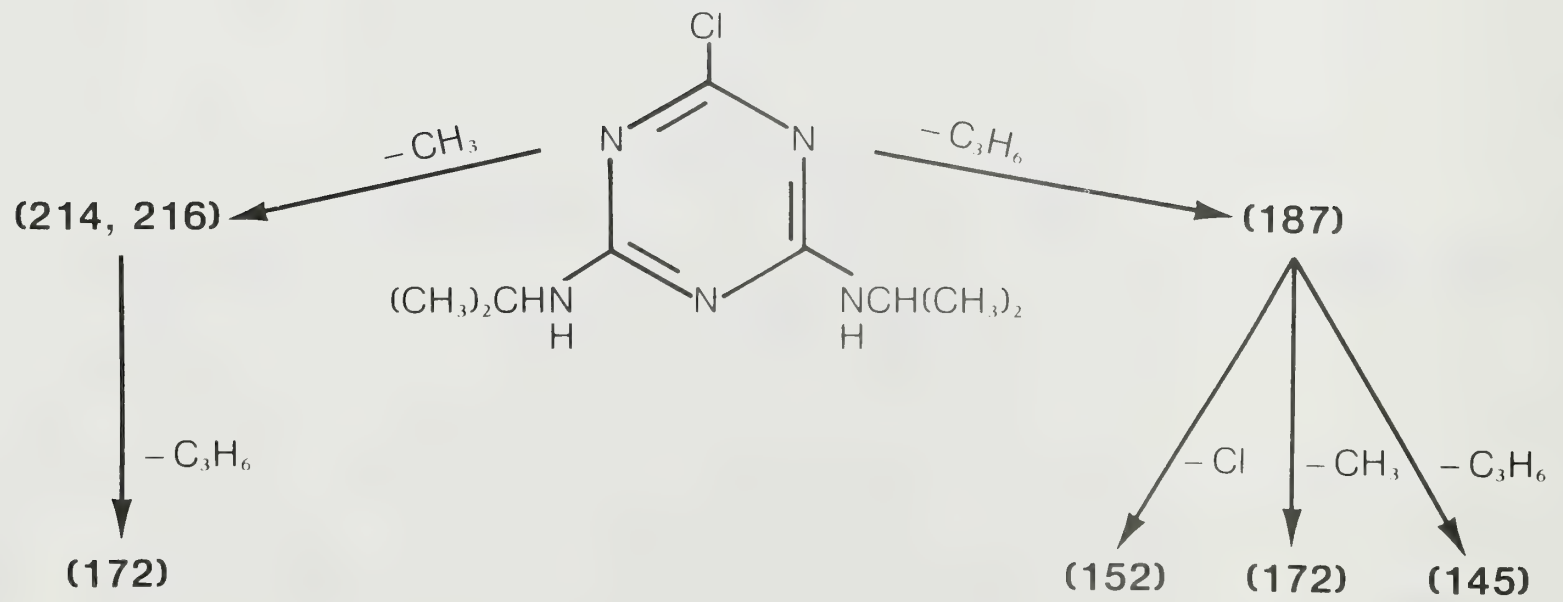
II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

TERBUTHYLAZINE
(229,231)

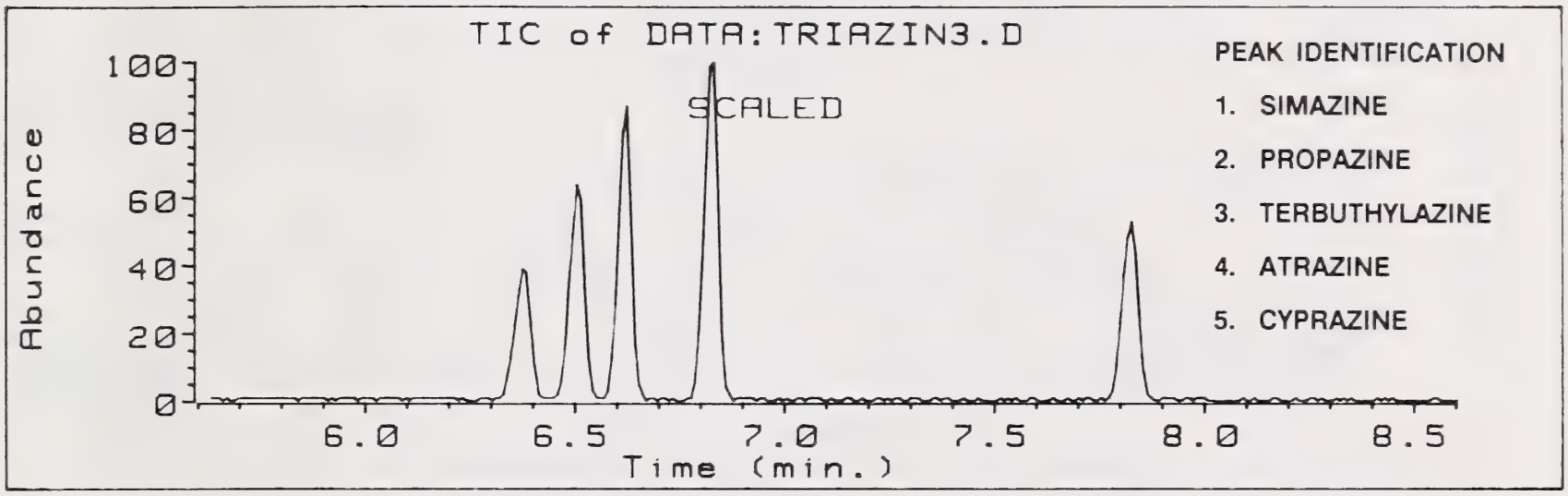


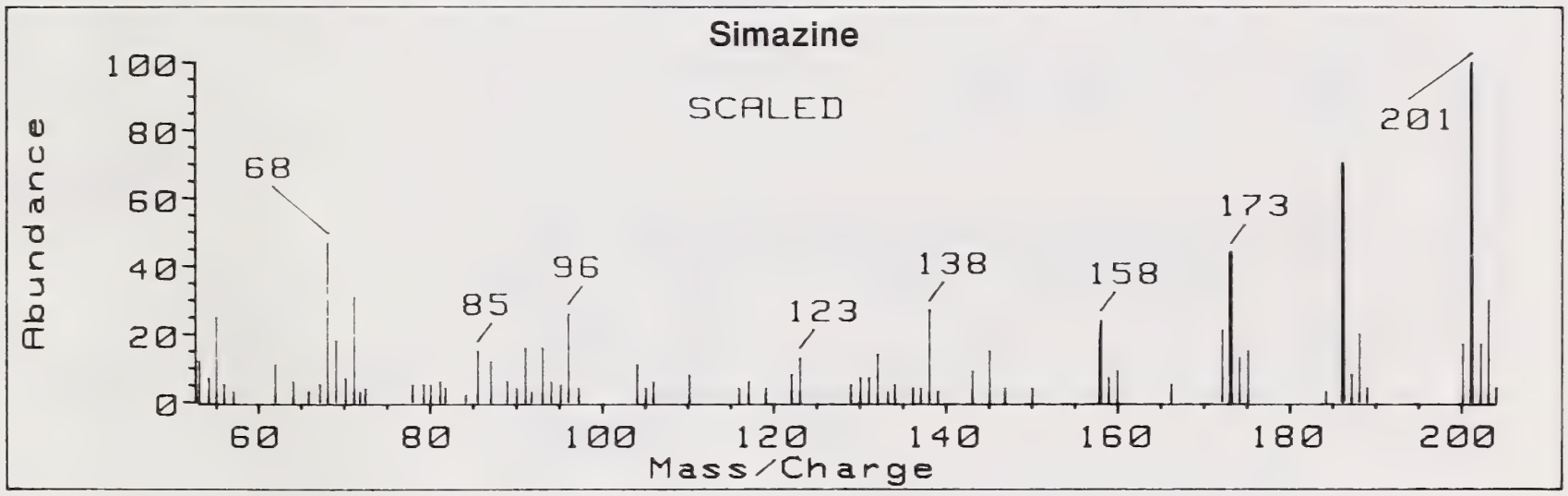
PROPAZINE
(229, 231)



II. CONFIRMATORY METHOD

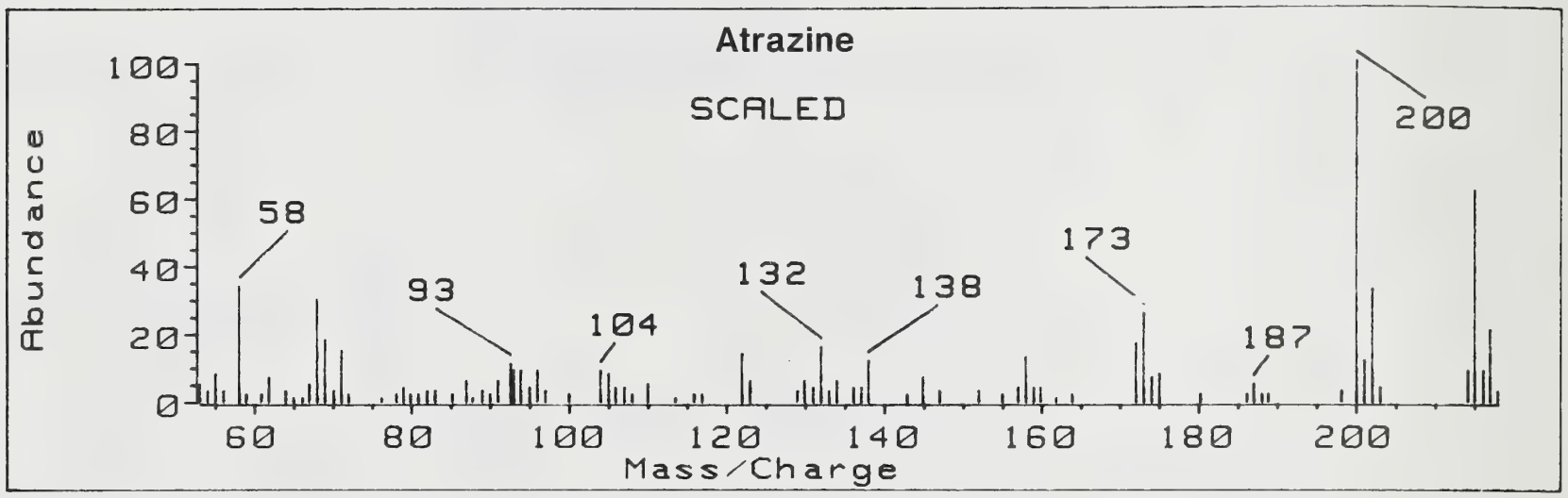
F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)

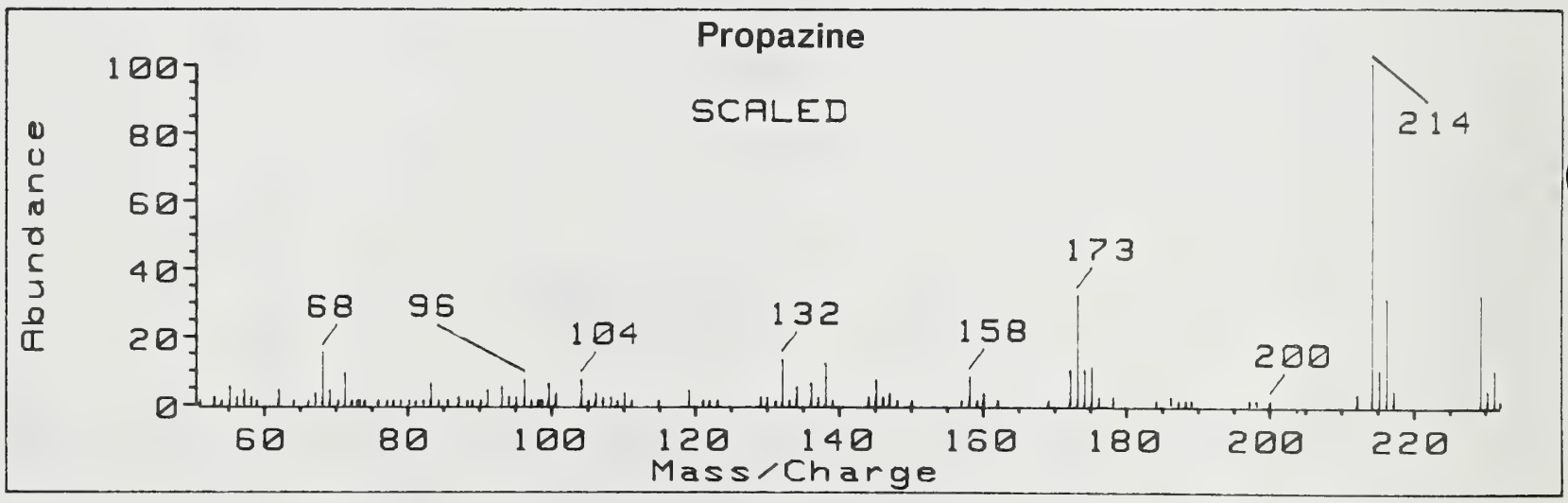


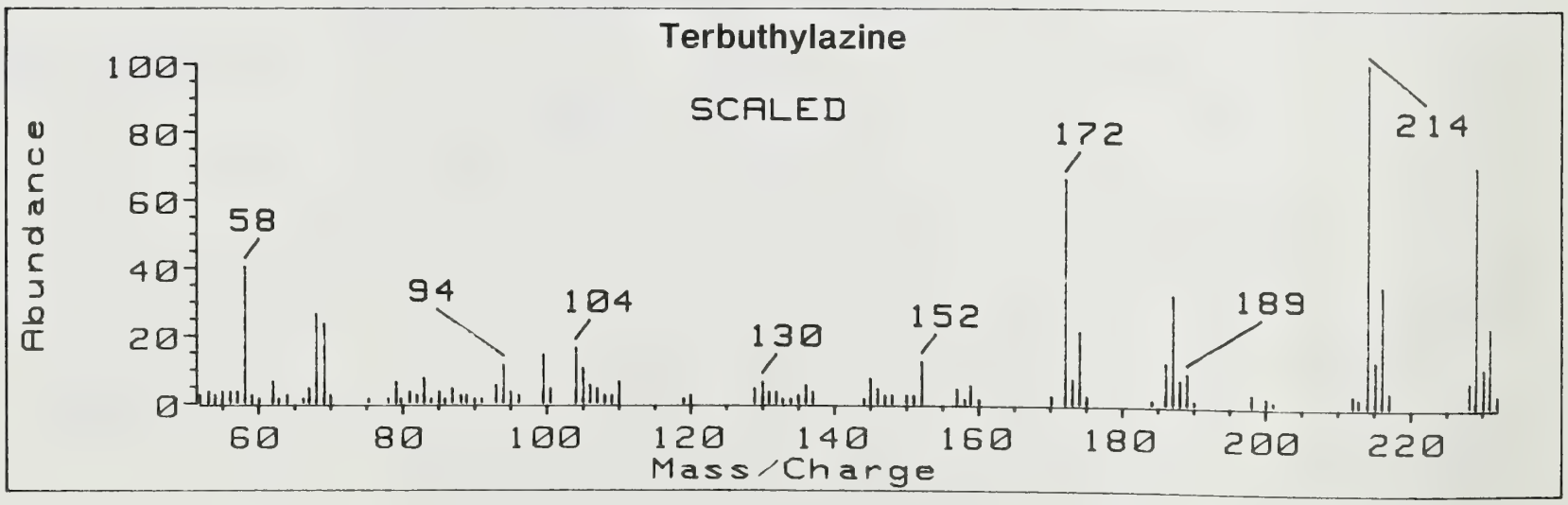


II. CONFIRMATORY METHOD

F. ANALYTICAL CONFIRMATION PROCEDURE (Continued)







II. CONFIRMATORY METHOD

H. HAZARD ANALYSIS

See Determinative Method, section H.

J. QUALITY ASSURANCE PLAN

1. Performance Standards

a. No false positives at 0 ppb for all compounds.

b. No false negatives at 10 ppb for all compounds.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	2.0 ± 0.1 g
b. Florisil weight	1.0 ± 0.05 g
c. Elution volume	15 ± 1.0 mL
d. Final extract volume	Dilute accordingly to match standard concentration

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—By at least 3 replicate injections of standards, show that instrument is functioning properly.

ii. Phase II: Minimum of 3 samples, with at least 2 positives, for all four triazines.

Submit data on standards and samples to Chemistry Division for information.

NOTE: Phase I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) Samples submitted by the supervisor.

(b) Report data to Chemistry Division, QSB.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

i. No false positives or negatives.

ii. Must monitor at least 6 ions.

iii. Four out of the six ions must show ion peaks free of significant interferences in the area where the analytes elute.

iv. Retention time of analyte peak must match the retention time window of the standard or fortified standard ± 2%.

v. Ion ratios for at least 2 ion pairs must match those of the 20 ppb equivalent standard and fortified sample at 20 ppb (± 15%).

II. CONFIRMATORY METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: At least one check sample biweekly per analyst containing at least one of the triazines and using different species. At least one out of four of the positive samples is to be confirmed by mass spectrometry on a quarterly basis.

ii. Random replicates may be chosen by the supervisor or Laboratory QA Officer for QA samples over a period of one year.

iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

(a) All replicate findings.

(b) Control chart on difference between replicates.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses for that analyst.

ii. Investigate and identify probable cause.

iii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Fat.

b. Sample receipt size, minimum: 50 g.

c. Condition upon receipt: Cold (< 10° C).

d. Sample storage:

i. Time: 6 months.

ii. Condition: −20° C.

6. Sample Set

Each set must contain a reagent blank, a tissue blank, a tissue blank fortified at 20 ppb, and all positive samples found above the concentration of interest from the determinative method.

7. Sensitivity

a. Lowest detectable level (LDL): Not applicable.

b. Lowest reliable confirmation (LRC): 10 ppb.

ZERANOL BY RADIOIMMUNOASSAY (RIA)

Contents

A. Introduction 1
B. Equipment 2
C. Reagents and Solutions 3
D. Standards 6
E. Extraction Procedure 7
F. Analytical Quantitation 9
G. Calculations 11
H. Hazard Analysis 14
I. [Reserved]
J. Quality Assurance Plan 15

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

A 1-gram sample of target tissue is homogenized, then hydrolyzed at 37° C for 2 hours with β-glucuronidase. The sample is then cleaned up by partitioning it between organic and aqueous phases, taking advantage of zeranol's acidic properties. After cleanup, RIA detection is employed, involving monoclonal antibodies raised against zeranol and calculated against a ten standard external curve. All samples are recovery corrected by use of an internal standard. The antibodies and methodology were developed by S. N. Dixon, K. L. Russel, and C. B. Millinson, UK.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

a. Polypropylene screw-top disposable centrifuge tubes, 50 mL.

b. Polypropylene screw-top disposable centrifuge tubes, 15 mL.

c. Polypropylene rimless disposable test tubes, 16 × 75 mm.

d. Separatory funnels, 1000 mL.

e. 37° C incubator.

f. LKB 1219 RACKBETA liquid scintillation counter, or equivalent.

g. RIA computer calculation facilities.

h. −20° C freezer.

i. Analytical balance.

j. Microscale tissue homogenizer.

k. Vortex mixer.

l. Magnetic stirrer.

m. Tilt measures or dispenser for 5 and 10 mL.

n. 10 mL repipette for scintillation cocktail.

o. 4° C refrigerator.

p. N-Evap analytical evaporator, or equivalent.

q. Eppendorf repeating pipette with 100 and 500 μL tips, or equivalent.

r. Eppendorf 10 to 100 μL adjustable pipette, or equivalent.

s. Eppendorf 100 to 1000 μL adjustable pipette, or equivalent.

t. Class A volumetric pipets: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mL.

u. Volumetric flasks with stoppers, 100 mL.

v. Bath for crushed ice.

w. Refrigerated centrifuge (4° C).

x. Rotors and carriers for 50 mL, 15 mL, and 16-75 mm RIA polypropylene tubes.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent List

a. 1.0N NaOH (VWR Scientific, Inc., reagent grade).

b. NaH_2PO_4, anhydrous (MCB, reagent grade).

c. Na_2HPO_4, anhydrous (J. T. Baker, reagent grade).

d. NaCl (EM Science, reagent grade).

e. Thiomersal [C_2H_5-HgS-C_6H_4-$COON_a$] (Sigma Chemical Co.).

f. Gelatin powder (J. T. Baker).

g. Norit SG II activated charcoal (MCB).

h. Dextran low fraction (Eastman Kodak).

i. 3H-Zeranol isotope (Amersham International—custom order).

j. Methanol (Burdick & Jackson).

k. β-Glucuronidase (Sigma Chemical Co., G-0876, type H-2_1).

l. Methyl-t-butyl ether (Burdick & Jackson).

m. Beckman Ready-Solve for aqueous samples, or equivalent liquid scintillation cocktail (Beckman order #566766).

n. Acetone, low grade (for dry ice bath).

o. Dry ice (solid CO_2).

p. Nitrogen (gas for evaporation).

q. Distilled water.

r. Zeranol monoclonal antibodies.

NOTE: Zeranol, 3H-Zeranol isotope, and Zeranol monoclonal antibodies may be purchased in a kit from Food & Veterinary Laboratory, Ltd., 25-26 Frederick Sanger Road Guildford, Surrey, UK, GU25YD, Tel. 011-0483-300443; FAX 011-0483-301171.

s. Chloroform (Burdick & Jackson).

t. Glacial acetic acid (Fisher reagent grade).

2. Solution List

a. Phosphate buffered saline (PBS).

i. 18.72 g NaH_2PO_4.

ii. 34.64 g Na_2HPO_4.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

iii. 36.00 g NaCl.

iv. 0.40 g Thiomersal [C_2H_5-HgS-C_6H_4-$COON_a$].

v. Make to 4.0 L with distilled water.

vi. Adjust to pH 7.0 with NaOH and HCl; store at 4° C.

b. Phosphate-buffered saline gelatin (PBSG).

i. Add 1.0 g/L gelatin powder to PBS (solution 2.a).

ii. Warm to dissolve and store at 4° C.

c. Charcoal solution.

i. 5.0 g Norit activated charcoal.

ii. 0.5 g Dextran low fraction.

iii. Make to 800 mL with distilled water and store at 4° C.

iv. Stir for at least 10 min in ice bath before use.

NOTE: Solution must be at 4° C when used.

d. Antiserum.

Dilute 1 to 80 in PBSG (solution 2.b) as needed.

NOTE: Leave one tube thawed and do not refreeze.

e. Isotope.

i. Working solution.

(a) Dilute 100 μL stock solution to 5 mL in methanol.

(b) Store at −20° C.

ii. Assay solution (Standard Addition "SA").

(a) Evaporate 70 to 100 μL working solution to dryness at 40° C, under nitrogen.

(b) Dilute to approximately 10,000 cpm/100 μL with PBSG (check on scintillation counter).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

iii. Recovery solution (Recovery Addition ''RA'').

Dilute assay solution to 1500 cpm/100 μL with PBSG (check on scintillation counter).

NOTE: Do not store aqueous assay or recovery solutions.

f. β-Glucuronidase.

Dilute stock Sigma solution (G-0876, type H-2) 1 to 10 with distilled water.

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Zeranol can be purchased from:

Pittman-Moore
Terre Haute, IN

or as part of a kit (see section C).

2. Preparation of Standards

a. Stock solution A.

i. If sufficient material is available, weigh 100 mg of pure zeranol and dissolve in 100 mL of methanol (i.e., make up to 100 mL using a volumetric flask).

Solution A = 100 mg/100 mL
= 1 mg/mL

ii. If insufficient material is available, weigh 10 mg of pure zeranol and dissolve in 10 mL of methanol (i.e., make up to 10 mL using a volumetric flask).

b. Stock solution B.

Pipet 1 mL of solution A and make up to 100 mL with methanol using a 100 mL volumetric flask.

Solution B = 1 mg/100 mL
= 1000 μg/100 mL
= 10 μg/mL

c. Stock solution C.

Pipet 1 mL of solution B and make up to 100 mL with methanol using a 100 mL volumetric flask.

Solution C = 10 μg/mL
= 10000 ng/100 mL
= 100 ng/mL

d. Standard curve solutions.

To prepare 100 mL of each of the following standards containing: 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 pg/0.1 mL methanol, pipet:

10 mL solution C, make up to 100 mL with methanol = 1000 pg/0.1 mL
9 mL solution C, make up to 100 mL with methanol = 900 pg/0.1 mL
8 mL solution C, make up to 100 mL with methanol = 800 pg/0.1 mL
7 mL solution C, make up to 100 mL with methanol = 700 pg/0.1 mL
6 mL solution C, make up to 100 mL with methanol = 600 pg/0.1 mL
5 mL solution C, make up to 100 mL with methanol = 500 pg/0.1 mL
4 mL solution C, make up to 100 mL with methanol = 400 pg/0.1 mL
3 mL solution C, make up to 100 mL with methanol = 300 pg/0.1 mL
2 mL solution C, make up to 100 mL with methanol = 200 pg/0.1 mL
1 mL solution C, make up to 100 mL with methanol = 100 pg/0.1 mL

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

Sample Extraction

a. Weigh 1 g of tissue into 50 mL screw-cap polypropylene tubes. Analyze two blank tissues and two spiked tissues with each analytical set.

b. Add 2 mL distilled water to each sample.

c. Prepare recovery addition (solution C.2.e.iii, 1500 cpm/100 μL). Add 100 μL to each of two scintillation vials. Add 10 mL scintillation cocktail to each of the two vials, and save both for the final count. (These recovery additions [RA] will be used in the calculations.)

d. Add 100 μL recovery counts to each sample weighed in step a above.

e. Homogenize for 1 minute. Wash homogenizer with 3 mL distilled water into tissue extract (per sample).

f. Add 100 μL β-glucuronidase to each sample and shake gently by hand for 10 sec. Incubate 2 hr at 37° C. Mix gently at 30-min intervals.

g. Add 10 mL methyl-t-butyl ether and cap and vortex each tube.

h. Centrifuge at 1600 G for 10 min at 20° C and freeze overnight in a −20° C freezer.

i. Decant ether layer into a conical 15 mL screw-cap polypropylene centrifuge tube.

j. Thaw the tissue extract (*Do not heat above 40° C.)* and add an additional 5 mL methyl-t-butyl ether.

k. Cap and vortex each tube and centrifuge at 1600 G for 10 min at 20° C.

l. Refreeze in a dry ice/acetone bath for 10 min, pool the ether extracts, and evaporate to dryness under nitrogen at about 40° C. *(To avoid losing zeranol in this step, remove the dry tubes from the 40° C bath immediately, as zeranol may adhere to the tubes and/or oxidize.)*

m. Redissolve in 1 mL redistilled chloroform (2 mL may be used if tissue is fatty) and vortex. *(Vortex to dissolve the fat before NaOH is added in the next step.)*

n. Add 3 mL 1N NaOH. Cap, vortex, and centrifuge for 10 min at 900 G at room temperature.

o. Transfer the NaOH layer *(taking care to transfer none of the chloroform layer)* to a 50 mL screw-cap polypropylene centrifuge tube containing 250 μL of 90% acetic acid, using a 1000 μL Eppendorf pipette.

p. Add 5 mL methyl-t-butyl ether. Vortex, freeze in dry ice/acetone bath, and decant ether layer into a conical polypropylene centrifuge tube.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

q. Evaporate ether to dryness and immediately add 1 mL PBSG (solution C.2.b). Gently rotate the tube to dissolve zeranol in gelatin buffer as foaming occurs if shaken vigorously. *Let stand a minimum of 30 min to ensure the zeranol has dissolved sufficiently. (Failure to do this can be a source of low recoveries.)*

r. Transfer 250 μL to a scintillation vial and 500 μL to a 16 × 75 mm rimless polypropylene tube.

   i. To the scintillation vial, add 10 mL scintillation cocktail and save (to be used in calculating results).

   ii. The 500 μL portion is to be used in the zeranol RIA.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Zeranol RIA

a. Standards.

i. Pipette 100 μL standards in sequence into 16 × 75 mm poly-proylene rimless tubes as indicated in assay tube order.

ASSAY TUBE ORDER

Tube #	Buffer μL	Isotope μL	Antiserum μL	Standard pg	Unknown #	Charcoal μL
1,2	500	100	100	—	—	—
3,4	600	100	—	—	—	500
5,6	500	100	100	0	—	500
7,8	500	100	100	100	—	500
9,10	500	100	100	200	—	500
11,12	500	100	100	300	—	500
13,14	500	100	100	400	—	500
15,16	500	500	100	500	—	500
17,18	500	500	100	600	—	500
19,20	500	500	100	700	—	500
21,22	500	500	100	800	—	500
23,24	500	500	100	900	—	500
25,26	500	500	100	1000	—	500
27,28*	500*	100	100	—	1,2	500
29,30*	500*	100	100	—	3,4	500
31,32*	500*	100	100	—	5,6	500

and so forth for all tissue samples.

*Sample

NOTES: (1) Tubes 1 and 2 are used in the calculations (SA) and 500 μL water is added instead of charcoal; (2) Tubes 3 and 4 are used in the calculations for nonspecific binding (NSB) and no antiserum is added; (3) Tubes 5 and 6 contain no zeranol and are used in the calculations as zero standards (B_o); (4) Tubes 27 and 28 are the blank tissue samples and begin the use of 500 μL samples from step r above instead of pure buffer.

ii. Evaporate to dryness under nitrogen at about 40° C and redissolve immediately in 500 μL PBSG. (Tubes 3 and 4 [NSB] receive 600 μL PBSG to compensate for the 100 μL not added to these tubes.)

b. Samples.

Label the 500 μL samples from step r according to the format in the assay tube order (beginning with tube #27).

c. Add 100 μL of the 10,000 cpm 3H-zeranol to each tube.

d. Add 100 μL antiserum to each tube except tubes 3 and 4 (NSB).

e. Mix gently (do not allow protein to foam) and incubate at 4° C overnight.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

NOTE: From this point until samples are decanted into scintillation vials, all samples and solutions are kept at 4° C.

f. Stir charcoal in ice bath for 10 min and precool centrifuge to 4° C.

g. Add 500 μL water to tubes 1 and 2. Add 500 μL of dextran-charcoal to all tubes (except tubes 1 and 2) within 2 min and incubate at 4° C for 10 min from the first addition of charcoal.

h. Centrifuge at 4° C for 10 min at 900 G immediately upon completion of 10-min incubation.

i. Place all tubes in an ice bath and decant supernatant to a scintillation vial.

j. Add 10 mL scintillation cocktail (Beckman Readysolve for aqueous samples, or equivalent) and count for 4 min or 10,000 counts on a beta scintillation counter.

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

a. Definitions.

i. B = Labeled zeranol bound to antibody, expressed in cpm per tube.

ii. B_o = Total labeled zeranol bound to antibody in the absence of any unlabeled zeranol, expressed in cpm per tube.

iii. NSB = Counts bound nonspecifically (nonspecific binding).

iv. SA = Standard addition (the total cpm added to each RIA assay tube).

b. Assumptions.

i. B/B_o for a given mass is constant over a narrow range of SA.

ii. The NSB is constant for each tube.

c. Standard curve.

Plot percent B/B_o (taking NSB into account) on the Y axis vs the mass of each standard (std) in picograms (pg) along the X axis.

$$(B/B_o)\% = \frac{(\text{cpm/std}) - \text{NSB} \times 100}{(\text{cpm/zero std}) - \text{NSB}}$$

NOTE: The curve is logarithmic.

d. Logit-Log analysis.

The Logit-Log analysis is used to linearize the logarithmic standard curve (often referred to as the dose response curve) by performing logit and log transformations on the Y and X axis respectively.

NOTE: A computer is recommended for the Logit-Log analysis.

i. Logit transformation: (Y axis).

$\text{Logit}(Y) = \ln [Y/(100 - Y)]$.

$Y = (B/B_o)\%$ ranging from 0 to 100 percent.

ln = Natural log (base e).

ii. Log(base 10)X:(X axis).

X = picograms zeranol per standard.

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

iii. When logit-log curve fitting is performed by hand, the use of logit-log graph paper is recommended. Available from TEAM, Inc., Tamworth, NH, or Codex, Norwood, NJ, or equivalent.

e. Tissue samples.

i. CPM/tube is corrected for CPM added as recovery cpm, SA, and NSB.

(a) $SA_{corr} - SA + [x(\text{recovery cpm})]$

$$x = \frac{\text{volume of sample used in RIA assay}}{\text{volume of sample used in recovery cpm}}$$

Recovery cpm (recovery counts) from step E.r.ii.

(b) $B_{corr} = \frac{B(SA)}{SA_{corr}}$

(c) Correction for NSB.

$$Y = [(B_{corr} - NSB)/(B_o - NSB)]100$$

(d) Read X (pg per tube) from the standard curve using Y or perform the logit transformation on Y and read X from the logit-log graph.

ii. The amount of zeranol found in each tube (pg per tube) must be corrected for the blank tissue value, the weight of each tissue, and the percent recovery.

(1) Water blank correction—Subtract pg of zeranol calculated for the blank tissue from each sample.

$V_t = W_b$ = Water blank correction

V_t = Amount of zeranol in pg from standard curve for each sample.

W_b = Amount of zeranol in pg from standard curve for blank tissue.

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

(2) Weight correction—Because only half the final 1-mL sample is used in the RIA, the results are multiplied by 2.

(3) Recovery correction—Because 0.25 mL of the final 1.0 mL is used for recovery cpm, the recovery cpm is multiplied by 4. The result is then divided by the total recovery addition (RA) from step E.d to yield the percent recovery.

% recovery = (recovery CPM)(4)(100)/RA

(4) Final pg correction formula corrections from ii.(1), (2), and (3) combined yield:

($V_t = W_b$)(2)(100/% recovery) = corrected pg/g = picograms of zeranol found in the one-gram tissue sample.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title

Determination of Zeranol by Radioimmunoassay (RIA)

2. Required Protective Equipment

Safety glasses, lab coat, disposable gloves. (Gloves should be worn at all times while handling tritiated compound.)

3. Procedure Steps

	Hazards	*Recommended Safe Procedures*
C. Reagents		
Methanol Acetone Chloroform Methyl-t-butyl ether	The organic reagents are very flammable, corrosive, and the vapors are very irritating to the skin, eyes, and respiratory system.	These solvents should only be used in an efficient fume hood, away from any heat generating devices.
1.0N NaOH Glacial acetic acid	Fumes are corrosive. Spattering may result in serious skin, eye, or respiratory damage.	Use in fume hood.
Tritiated zeranol	Cancer radiation risk.	Gloves should be worn at all times while handling.
Thiomersal	Poison, skin irritant.	See above.

4. Disposal Procedure

Organic solvent mixture	See above.	Segregate chlorinated from nonchlorinated solvents. Hold in designated containers until disposed of by contractor or in-house specialist.
Tritiated zeranol waste	See above.	Tritiated zeranol waste may be burned in an EPA-approved incinerator.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppt)*	*Acceptable Recovery (%)*	*Repeatability CV %*
Zeranol	100-1000	80-100	< 20

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Refreeze in a dry ice/acetone bath.	Remove 15 mL tubes from N-evap as soon as dry.
b. Transfer the NaOH layer.	Transfer none of chloroform layer in NaOH/$CHCl_3$ mix. Monoclonal antibodies will not combine with zeranol in the presence of $CHCl_3$.
c. Transfer from centrifuge tube.	From this point until samples are decanted into scintillation vials all samples and solutions are kept at 4° C.

3. Readiness To Perform

a. Familiarization.

i. Phase I—Standards—RIA Zeranol 10-point standard curve from 100 ppt to 1000 ppt in triplicate.

ii. Phase II: Self-fortified tissue recoveries—Zeranol 10-point tissue curve from 100 ppt to 1000 ppt in triplicate. Use tissue commonly analyzed for zeranol (supervisory discretion).

iii. Phase III: Check samples for analyst accreditation.

(a) 16 samples submitted by supervisor.

(b) Report analytical findings to Chemistry Division.

b. Acceptability criteria.

Phases I and II correlation coefficient of .990 or better Phase III CV < 20%.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: 1 per week per analyst.

ii. Records are to be maintained by analyst and reviewed by supervisor and Laboratory QA Officer for:

(a) Determined value versus known value; maintain CUSUM.

(b) Percent recovery.

b. Acceptability criteria.

i. No false positives or negatives.

ii. % recovery not to exceed 20% CV.

iii. If unacceptable values are obtained, then:

(a) Stop official analysis.

(b) Investigate and identify probable cause.

(c) Take corrective action.

United States Department of Agriculture

Food Safety and Inspection Service

Washington, D.C.
20250

OFFICIAL BUSINESS
Penalty for Private Use, $300

SPECIAL FOURTH CLASS